THE PREPARATION AND STUDY [redacted]
NEUROTOXIC ORGANIC PHOSPHA[redacted]

ABSTRACT

THESIS SUBMITTED FOR THE DEGREE OF

Doctor of Philosophy

IN

CHEMISTRY

TO

THE ALIGARH MUSLIM UNIVERSITY, ALIGA[redacted]

BY

SYED FATEHYAB ALI

T2137

DEPARTMENT OF CHEMISTRY
ALIGARH MUSLIM UNIVERSITY
ALIGARH

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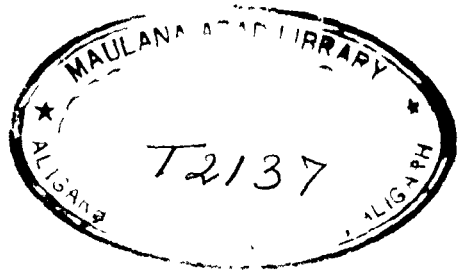

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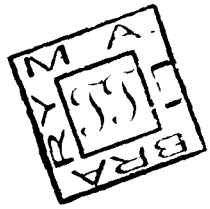

C E R T I F I C A T E

I certify that the research work incorporated in this thesis entitled "The preparation and study of neurotoxic organic phosphates" has been carried by Syed Fatehyab Ali and that it is suitable for the award of Ph.D. degree in Chemistry of the Aligarh Muslim University, Aligarh.

(Dr. Arif Zaman)
Reader,
Department of Chemistry,
Aligarh Muslim University,
Aligarh-202001 (INDIA).

TABLE OF CONTENTS

2. MATERIAL AND METHOD

LIST OF FIGURES

LIST OF TABLES

A C K N O W L E D G E M E N T

I wish to express my deep sense of gratitude to Dr. Arifuzzaman, Reader, Department of Chemistry and Professor Dr. Mahdi Hasan, Brain Research Center, Jawaharlal Nehru Medical College, Aligarh Muslim University, Aligarh, for their valuable guidance and encouragement throughout thir work.

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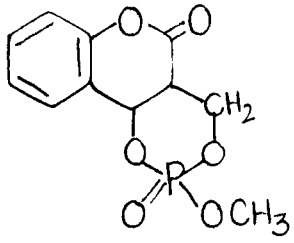
Lastly, I am extremely thankful to Council of Scientific and Industrial Research (CSIR), New Delhi for the financial assistance in the form of a Senior Research Fellow.

S. Fatehyab Ali

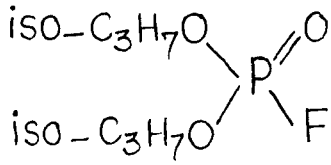
(SYED FATEHYAB ALI)

A B S T R A C T

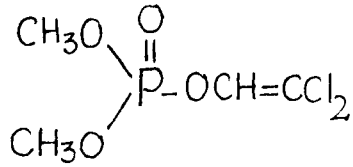
Organophosphates are powerful, widely used pesticides. Their chemistry and biological activities are therefore of much practical interest and are discussed briefly in this thesis. Two organophosphorus compounds (I and II) were prepared during the course of this work, of which one is new and has structure (I). It was prepared from 4-hydroxycoumarin which is a unit of dicoumarol a coumarin dimer used as an anticoagulant. This work was combined with a study of neurotoxicity of dichlorvos (III).



(I)



(II)



(III)

Dichlorvos (0,0-dimethyl 2,2-dichlorovinyl phosphate) was administered i.p. 0.6, 1.5 and 3.0 mg/kg body weight, daily for 3-15 days to a group of albino rats for recording the behavioural changes (open field behaviour and locomotor activity) and for biochemical study (acetylcholinesterase activity, concentration of putative neurotransmitters, levels of catecholamine (DA and NE) and 5-HT and the rate of lipid peroxidation). Further, their brains were prepared for histochemistry (acetylcholinesterase, cytochrome oxidase and succinic dehydrogenase) and for electron microscopy (perfusion-fixation).

The behavioural changes of the present studies revealed that the open field behaviour was significantly depressed below the mean of the control group. On the seventh day ambulation was reduced to 24% of the mean but recovered to 60% on the tenth day. Similarly, rearing response was decreased on the seventh day and showed a fast recovery on the tenth day but the preening response further declined on the tenth day. Defecation, on the contrary, was suppressed to zero per cent on the seventh day and showed complete recovery on the tenth day. Locomotor activity showed a significant depression and fine movements were reduced more than the gross movements in the second phase.

Dopamine was significantly decreased on the fifth and seventh day but showed a 13% recovery in the brain stem on the tenth day. Norepinephrine was significantly reduced in the cerebral hemisphere while 5-HT was decreased both in cerebral hemisphere and brain stem. Neither of these two amines showed significant recovery on the tenth day. Interesting concordance was found of the open field behaviour changes with the levels of dopamine, norepinephrine and 5-HT in various regions of the rat brain.

The neurotoxic effects of dichlorvos were also seen after 15 days of intraperitoneal administration to rats and were associated with significant lowering of taurine, GABA, glycine, lysine, phenylalanine and aspartic acid concentration of different regions of the central nervous system; cerebral hemisphere, cerebellum, brain stem and cervical spinal cord. Diminution of serine level was insignificant. The results suggest that in dichlorvos-induced

lowering of putative neurotransmitters amino acid concentration varies in different discrete regions of the brain and spinal cord. The significant diminution of these amino acids is likely to explain some of the toxic effects of dichlorvos.

The main findings of the electron microscopic study of the hypothalamus, hippocampus, cerebellum and spinal cord following dichlorvos administration (3 mg/kg body weight, i.p. daily for 10 days) supported with histochemical and quantitative biochemical estimation of acetylcholinesterase activity and the rate of lipid peroxidation were as follows:

1. A dose-dependent inhibition of the acetylcholinesterase activity was noted.

2. A dose-dependent increased of the rate of lipid-peroxidation was noted.

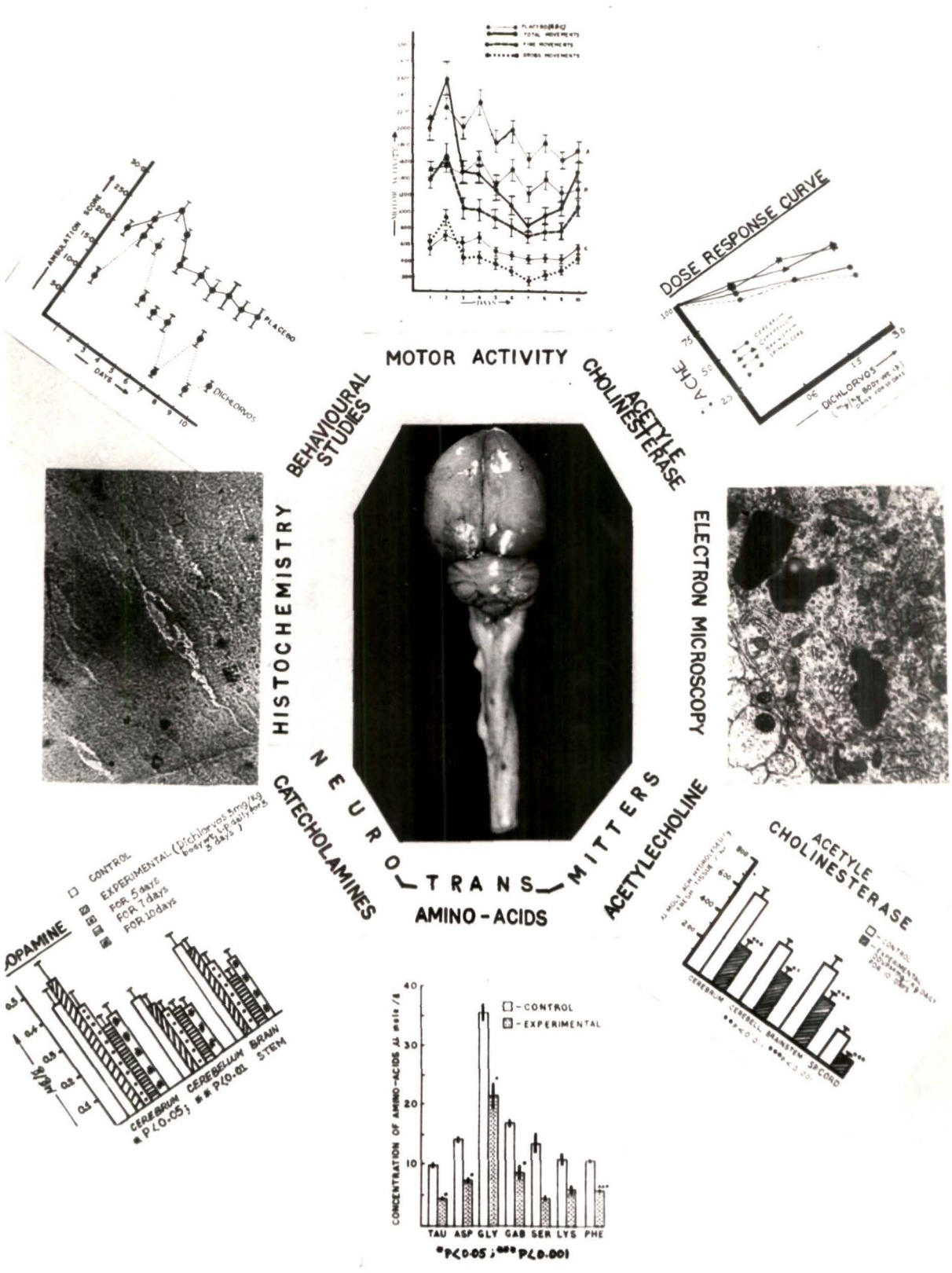
3. Evidence of edema around capillaries and axon terminal was observed.

4. A large number of electron dense bodies exhibiting electron-lucid vacuoles were discernible in the perikarya of cerebellar neurons.

5. Remarkable increase in the electron density of some axonal profiles and the occurrence of myelin-figures in a few axons and dendrites were detected.

6. Electron dense bodies were significantly increased in the neuronal perikarya of the spinal cord.

E.M. HITACHI 11E
LKB-ULTRATOME III
ORTHOPLAN & ORTHOMAT (LEITZ)
KRYOTOME 1310K (LEITZ)
ANIMEX ACTIVITY METER (LKB-FARAD- SWEDEN)
OPEN FIELD BEHAVIOUR APPARATUS
SPECTROPHOTOFLUOROMETER (CARL ZEISS)
SYNTHESIS & BIOCHEMICAL
DICHLORVOS


MOTOR ACTIVITY
BEHAVIOURAL STUDIES
ACETYLE CHOLINESTERASE
HISTOCHEMISTRY
ELECTRON MICROSCOPY
NEURO_TRANS_MITTERS
CATECHOLAMINES
ACETYLECHOLINE
AMINO-ACIDS
DOSE RESPONSE CURVE
DICHLORVOS
PLACEBO
DAYS
CONTROL
EXPERIMENTAL
FOR 5 days
FOR 7 days
FOR 10 days
DOPAMINE
CEREBRUM
CEREBELLUM
BRAIN STEM
ACETYLE CHOLINESTERASE
CONCENTRATION OF AMINO-ACIDS
TAU ASP GLY GAB SER LYS PHE
*P<0.05 ; ***P<0.001

INTRODUCTION

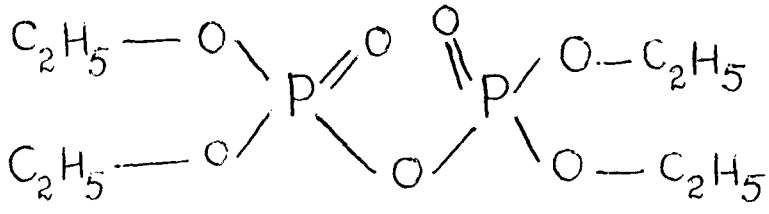
1. INTRODUCTION

1.1. Organophosphorus Compounds

Organophosphorus compounds are used as insecticides and pesticides for the control and destruction of organism interfering with human food, health and other amenities. The yearly production of organophosphate is now approaching 100,000 tons (Fest and Schmidt, 1973). The utility of the organophosphate pesticide is undisputed today: It can be expected that they will solve many of the world's nutritional problems. These compounds form the basis of a large number of insecticides, pesticides, nematocides and fungicides widely used in agriculture (for example dichlorvos (DDVP), malathion, parathion, thimet, metasystox, nuvacron, vydate, TEPP, baytex, chlorthion, guthion, EPN, delnar, dylox, ethion, fluorthion, rumithion, folithion, demeton, sytron, optunal, hinosan, ceresin, kitazin, conen, inejin, galecron etc.). Some organophosphates are also used as plasticizer in plastic industry or as a lubricant and additive e.g. tri-orthocresyl phosphate (TOCP), diazinon etc. According to the W.H.O. report of 1967, approximately, 1300 compounds have so far been prepared out of which some are not recommended for use due to their high level of mammalian toxicity. The statistics for 1969 indicates that 50 to 90% of the poisons detected in medicolegal cases belong to organophosphorus and organo-chlorine group of pesticides (Jani, 1971). Therefore, the increasing use of the organophosphate insecticides pesticides offers an important example of applied research.

1.2. Historical Account:

1.2.1. Synthesis: In 1820 Lassaigne for the first time reacted alcohol with phosphoric acid in a reaction analogous to that with sulfuric acid and thereby launched the chemistry of the organophosphorus compounds. Later, in 1847 Thenard synthesized phosphines and in the same year Cloez (1847) discovered the synthesis of thiophosphoric acid ester. Clermont (1854) synthesized tetraethyl pyrophosphate (later it became known as TEPP) (I) by alkylating the silver salt of the pyrophosphoric acid with alkyl halides.

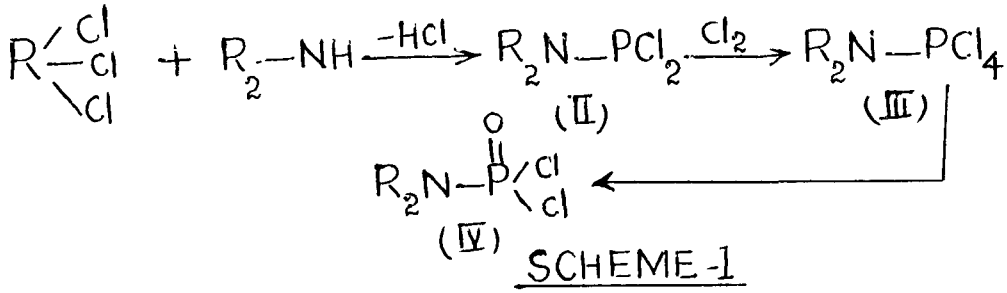


(I)

This ester (TEPP) is the bridge between the inorganic and organic chemistry. The insecticidal activity of this compound was discovered after 80 years of its synthesis. Hofmann in 1872 synthesized the corresponding phosphoric acid by reacting methyl and ethyl phosphine with nitric acid. Subsequently (1897) Michaelis and Becker synthesized a phosphonic acid ester by the reaction of dialkyl phosphite with ethyl iodide. This reaction became well known as Michaelis-Becker reaction.

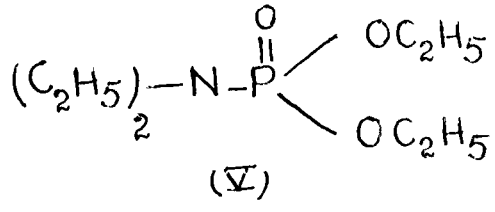
Michaelis and Kaehne (1898) synthesized another compound from trialkyl phosphite and methyl iodide whose structure did not resemble the compound which was obtained by Michaelis-Becker reaction.

In the early 19th century Michaelis repeated the reaction of phosphorus trichloride with sodium ethylate. At the same time Michaelis (1903) also synthesized phosphorus nitrogen compounds by the reaction of ammonia or amines with phosphorus trichloride, pentachloride, phosphoryl chloride and thiophosphorylchloride. He synthesized N-alkylamino-dichloro phosphine (II) by the reaction of phosphorus trichloride with alkylamine, which he oxidised with chlorine to the tetrachlorides (III) and atmospheric moisture sufficed to hydrolyze the tetrachlorides to dialkylaminophosphoro-dichloridates (IV).



SCHEME-1

He also synthesized O,O-diethyl N, N-diethyl phosphoroamidate (V) and O-ethyl N,N- diethyl phosphoroamidocyanidate (VI) by the reaction of N,N- diethyl phosphoroamidodichloridate with potassium cynide in absolute alcohol.



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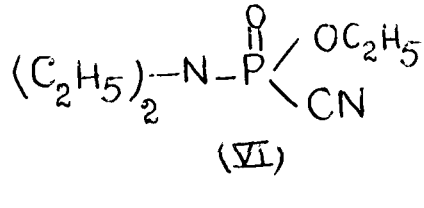
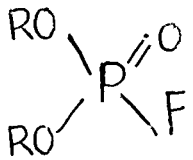
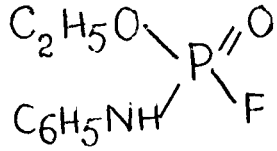


(VI)

In 1932 Lange and Krueger for the first time prepared the ester of monofluorophosphoric acid by the reaction of silver flurophosphate with alkyl halide (VII, VIII).



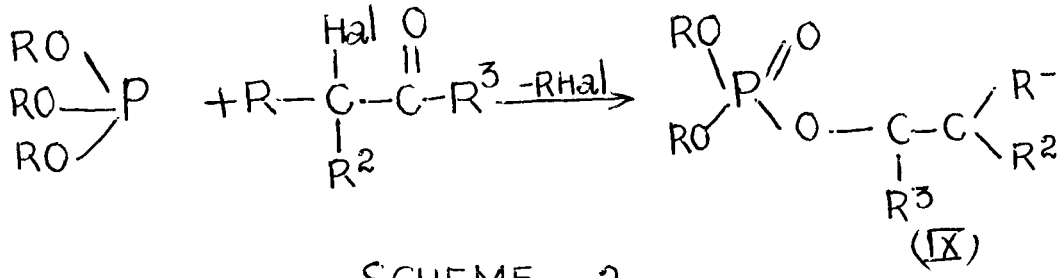
(VII)



(VIII)

R = i-propyl
Sec.Butyl

They also reported the highly toxic properties of these compounds. The toxicity of fluorophosphoric acid manifested itself in the form of the following symptoms; respiratory distress, clouding of the consciousness, temporary blindness and photophobia (Lange and Krueger, 1932). During the second world war Saunders and his colleagues (1941-1946) prepared a number of fluorophosphates. They also described the miotic and high inhalation toxicity of these compounds. In 1952-54 Perkow synthesized a number of dialkylvinyl phosphates (IX) by the reaction of triethyl phosphite with the α-halogen carbonyl compounds.

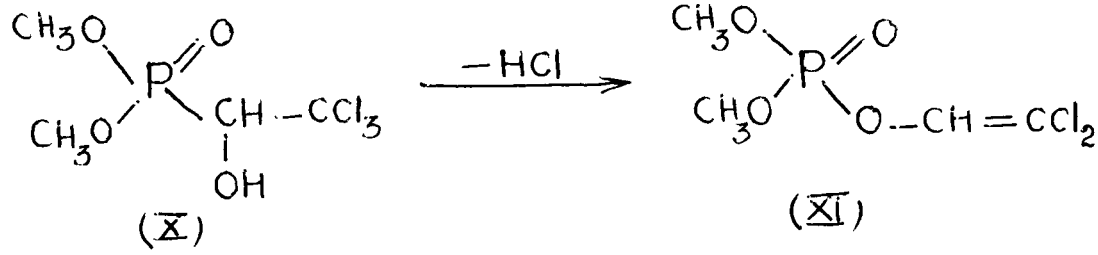


(IX)

SCHEME -2

To honour its discoverer the reaction is known as the Perkow reaction. Dichlorvos (dichlorovinyl phosphate) is one of the important products thus obtained. It is synthesized by treating of triethyl phosphite

with chloreal. A different rout to such compounds was discovered by Lorens (1954) who showed that trichlorfon (X) is hydrolyzed to dichlorvos (XI).



(X) (XI)

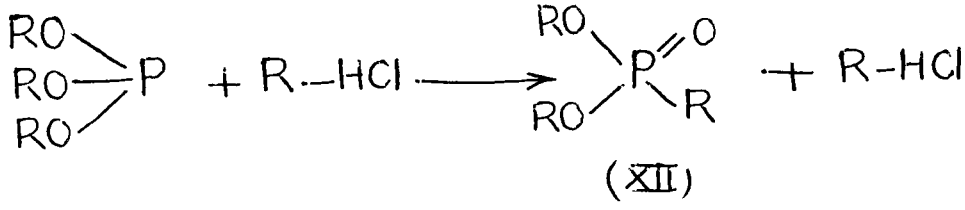
Mattron et al. (1955) reported that in the synthesis of Dipterex (trichlorfon), dichlorvos appears as a side product. Robin and Haar (1956) discovered a new method for the synthesis of trichlorfon and they also reported the rearrangement of trichlorfon to dichlorvos in alkaline condition.

1.2.2. Toxicity: In the last years of 19th century, phosphocreosote was used against pulmonary tuberculosis, but the high level of organophosphorus compounds generally restricted their use as chemotherapeutic agents. Thus Lorot (1894) reported 6 cases of paralysis out of 41 individuals treated with phosphocreosote (containing about 5-12% organophosphate). During the prohibition period of 1930 in the U.S.A., extract of ginger (U.S.P.) was used as a beverage of its alcoholic content. At the same time an oil named lindol which contains tri-o-cresyl phosphate was in use in celluloid and varnish industry. Some 20,000 Americans were paralyzed in 1930 when ginger got adulterated with lindol (Aring et al., 1941; Aring, 1942). In 1932 apiol, a drug employed for illegal abortions, was found to causes paralysis in many cases due to its contamination with organophosphates. Eleven cases of paralysis were observed in Holland (Moeschlin, 1952) and sixty in Germany (Creutzfeldt and Orzechowsk, 1941-43). Another

outbreak of paralysis was reported from Durban in 1937 when cooking oil stored in drums used for lubricating oil, which contained O-cresyl phosphate, was consumed by the Africans. Later in 1940, 80 soldiers belonging to a unit of the Swiss Army were poisoned when machine oil containing organophosphates contaminated the cooking oil. A second outbreak of paralysis occurred in Durban (1957) when eleven Africans used water stored in drums taken from a paints factory. Two years later a major epidemic of toxic neuritis broke out in Morocco. Ten thousand persons were affected when cooking oil adulterated with a lubricating oil (containing 3% cresyl phosphate), which was used in jet planes, was accidentally consumed (Smith and Spalding, 1959). Other outbreaks of paralysis have been reported from different countries including England, Germany and India (Vora et al., 1962).

1.3. Nomenclature:

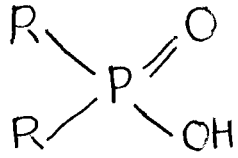
The nomenclature of the organophosphorus compound was not accepted easily. This was due to different groups of workers who gave the name of the compound after their own method. For example, a product (XII) which was formed by the reaction of trialkyl phosphite with alkyhalide as follows;



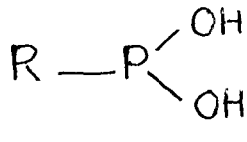
(XII)

SCHEME -III

It was termed as, phosphinic acid ester by Russians, while German workers designated it only as "Phosphonic acid". A survey of the literature, therefore, leads into difficulties due to this difference in terminology of the same compound adopted by the different authors. This anomaly was thus to start with in the report of German and Russian authors and Anglo Saxon literature of subsequent years is also full of it. As an illustration of the latter, let us examine the undermentioned compounds (XIII) & (XIV).



(XIII)



(XIV)

Whereas the Americans recognize (XIII) as phosphinic acid and (XIV) as phosphonous acid, the British workers designate (XIII) as phosphonour acid and (XIV) as phosphinic acid. It is thus apparent that the terminology adopted by the two schools is contradictory. German nomenclature is to some extent congruent with the American system. In the American nomenclature an acid with two P-C bonds is given the ending - inate in the pentavalent form while in case of trivalent the ending is inite. If there is only one P-C bond, it is designated as phosphonate/phosphonite. But in the case of English nomenclature proposed by IUPAC the names of these compounds have been reversed. The main disadvantage of IUPAC nomenclature is that it is of a philological nature and in other languages it is almost impossible to form words with analogous syllables. Therefore, in the nomenclature of organophosphorus compounds it is very difficult to combine all the international units. Consequently,

we have on the one hand Van Wazer's philosophy (1958) "..... what's in a name? That which we call a rose by any other name would smell as sweet" ..... (Romeo and Juliet II, 2) and on the other hand, we always use structural formulae when we discuss these problems.

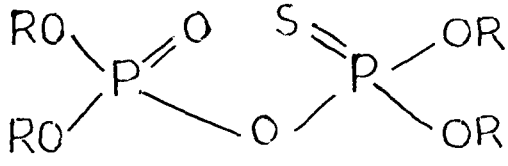
1.4. Spectroscopy in Organophosphorus Compounds:

As elsewhere in chemistry spectroscopic methods were made there impact in structure elucidation of organophosphorus compounds. A detailed discussion of the UV and IR spectra would be out of place here but it must be mentioned that more complex orbitals involved in the case of phosphorus compounds make it difficult to generalised on the basis of ultraviolet absorption in the same way as with carbon compounds. The most useful spectroscopic technique is nuclear magnetic resonance, mass spectroscopy providing easy access to the molecular weight of the compound.

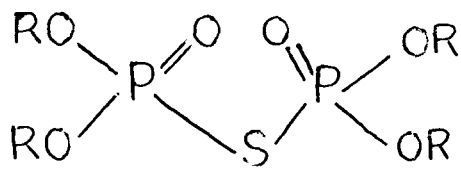
In the IR spectrum the group frequencies are generally the same as in other organic compounds but conjugation with phosphorus, causes marked changes in carbonyl absorption. Thus for example the carbonyl group of the yield of the β-keto group shows only weak absorption at 1600 cm^{-1}. The stretching frequencies of most functional groups have been determined and are reproduced here in tabulated form (Table-1).

While ^{31}P n.m.r. spectroscopy can offer useful information along with ^{1}H n.m.r. spectra, certain practical difficulties, which

need not be considered in detail here, restrict its application. ^{31}P nuclear magnetic resonance has been used to distinguish between trivalent and pentavalent phosphorus. Thus Ramirez (1964) has shown that the adduct of trialkyl phosphite and α-diketones have the structure (XV) rather than (XVI). Again repeate attempts to synthesized compounds with structure (XV & XVI) led to the same product the ^{31}P n.m.r. of which showed only one type of phosphorus



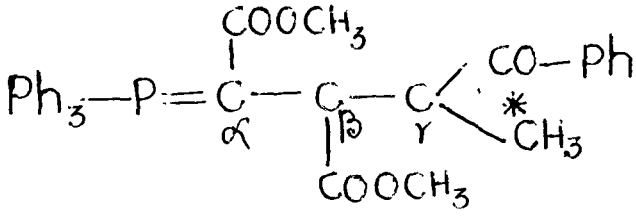
(XV)



(XVI)

suggesting that the symmetrical structure of (XVI) is correct.

The proton n.m.r. spectra are similar to those of organic compounds but the phosphorus to proton coupling is special feature here for example phosphorus in (XVII) shows a coupling with methyl group attached to the α-carbon atom.



(XVII)

Table - I : Characteristic infrared frequencies of Phosphorus - X bonds.

Bonds	Frequency range (cm^{-1})	Bonds	Frequency range (cm^{-1})
P - I	260 - 290	P - P	730 - 945
P - CF_3	460 - 460	P - C	700 - 935
P - S	410 - 470	P =	670 -1025
P - Si	385 - 505	PO_3	875.....950 - 935...1085-1160...1180
P - Br	240.....390 - 530.....575	PO_2	1000.....1040 -1100...1130-1220...1305
P - Se	400.....440 - 535.....580	P = O	1210 -1310...1410
P - Cl	340.....395 - 585.....650	P - H	1170.....1210 -1345...1390
P - C	650 - 780	P - Ph	1430 -1470
P - N	630 - 780	P - O	1645 -1790
P - S	530.....645 - 765.....835	P - H	2245 -2445
P - B	530 - 830		

1.5. Neurotoxicity of Organophosphorus Compounds:

As is well known, the toxic effects of organophosphates are due to the ability of these compounds to inhibit cholinesterase activity and to allow the accumulation of acetylcholine at nerve endings (Durham et al., 1957; Van Asperen, 1958; Holmstedt, 1959; Tracy et al., 1960; Casida, 1964; Goldstein et al., 1968; Polson and Tattersall, 1969). One of the best understood examples of drug-receptor interaction through formation of a covalent bond is the long lasting inhibition of cholinesterase by organic phosphates (Casida, 1964). Cholinesterase belongs to the class of "serine enzymes", which have a serine residue in the active centre that plays an essential role in the catabolism. In pseudocholinesterase, the aminoacid sequence in the active centre is glutamic acid-serine-alanine (Goldstein et al., 1968). In the hydrolysis of acetylcholine by cholinesterase, an extremely unstable acetyl derivative of cholinesterase is formed. The reaction with phosphates yield a more stable phosphorylated esterase (Casida, 1964). Phosphorylation, as by organophosphate, yield the following amino acid sequence at the esteritic site: glycaspartyl or glutamyl-phosphoryl-seryl-glycyl or alanyl.

According to Johnson (1976) when a neurotoxic dose of an organophosphorus ester is injected, a characteristic "neurotoxic protein" in nervous tissue is always phosphorylated "in vivo". The phosphorylation site is the active site of neurotoxic esterase. It is well known that the conformation and enzymatic activity of various proteins are reversibly modified by phosphorylation/ dephosphorylation reactions. It is possible that the monosubstituted

phospheryl residue, firmly bound at the active site of neurotoxic esterase, interfers with a portion of such a control mechanism (Johnson, 1976). Certain responses of the organism to organophosphate intoxication inhibition alone must also be considered. Carida (1964) has reported a delayed and prolonged neurotoxicity with associated demyelination and muscular weakness resulting from some organophosphates in several species, including man. More recently, Wadia et al. (1974) have described neurological finding in 200 consecutive cases of suicidal ingestion of organophosphorus insecticides. The authors found the following common signs: miosis, impairment of consciousness, muscular fasciculation, convulsions, toxic delerium and paralysis. Also Wadia et al. (1974) believe that the delayed neurotoxicity may represent an alternative mode of toxicity with organophosphorus compounds. Thus, not all neurological signs and symptoms of organophosphate intoxication can be explained on the basis of cholinesterase inhibition alone. The biochemical lesion leading to the typical delayed neurotoxicity has not been defined so far (Carida, 1964; Wadia et al., 1974).

1.6. Mechanism of Action of Organophosphorus Compounds:

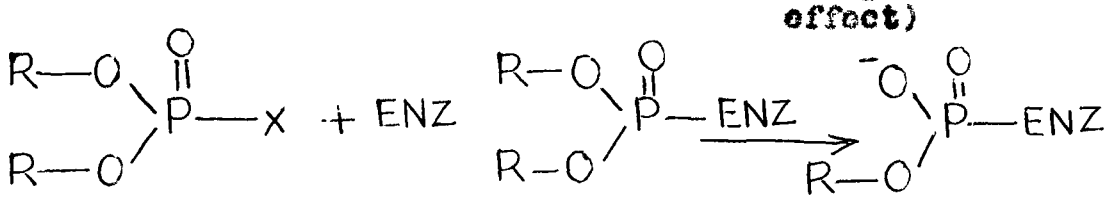
The biological action of organophosphorus compounds on mammals and insects is exerted by attaching the neural transmission system and thus interfering with the function of nervous system. Neuron is the basic structural and functional unit of the nervous system. It comprises of the nerve cell body together with all its processes. The processes carrying impulses towards

the cell body are called dendrites and one long process carrying impulses away from the cell body is known as an axone. Several neurons combine together to form a common functional pathway. This functional conjunction is established in the synapses. They consist of a presynaptic membrane (usually an axone or rarely a dendrite) and a post synaptic membrane (cell body a dendrite or occasionally an axone) and the distance between these two membrane is called synaptic gap which is about 100 - 200 Å. The impulse is transmitted in the synapses by a chemical mechanism. On the presynaptic side, acetylcholine, noradrenaline, dopamine, serotonin, GABA or any other neurotransmitter is released which later reacts with receptors on the post synaptic side, thus altering the permeability of this membrane to ions. In organophosphate poisoning the enzyme acetylcholinesterase is inhibited. Acetylcholinesterase is an enzyme which hydrolyses acetylcholine to acetic acid and inactive choline. Choline-O-acetyltransferase is another enzyme which esterifies both compounds to acetylcholine again. ATP and CoA are required for this reaction. Johnson (1976) reported that a variety of organophosphorus esters cause delayed neurotoxic effects in man, hen and other mammals. According to Johnson the neurotoxic inhibitor of neurotoxic esterases are phosphates, phosphonates or phosphoramidates. The substituted phosphoryl, phosphonyl, or phosphoramidyl enzyme derived from these inhibitors could hypothetically undergo a secondry reaction (analogous to "aging" of inhibited cholinesterase) to yield and acidic group bound to the enzyme as shown in Fig. 1. Phosphorylation/dephosphorylation reaction reversibly modified the conformation and enzymatic activity of various proteins. Monosubstituted

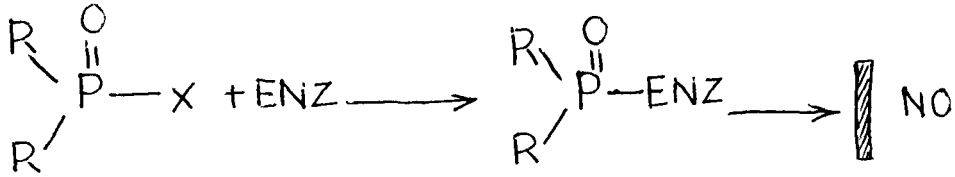
phosphoryl residue which is firmly bound at the active site of neurotoxic esterase interferes with a portion of such a control mechanism (Fig. 1). Activity of an enzyme turned "full on" or "full off" in the neuron could be disruptive while the fixation at "base line" by protective compounds might be tolerable atleast for a while (Johnson, 1976) Fig. 2.

ENZYME ⟶ INHIBITED ENZYME ⟶ "AGED" INHIBITED ENZYME

(Active esterase: in essential) (No esterase activity but no toxic effect) (Protein with extra charge: leads to toxic effect)



Phosphate (neurotoxic)



Phosphinate (protective)

Fig. 1: Comparison of suggested consequences of inhibiting neurotoxic esterase with a phosphate (neurotoxic) and a phosphinate (protective).

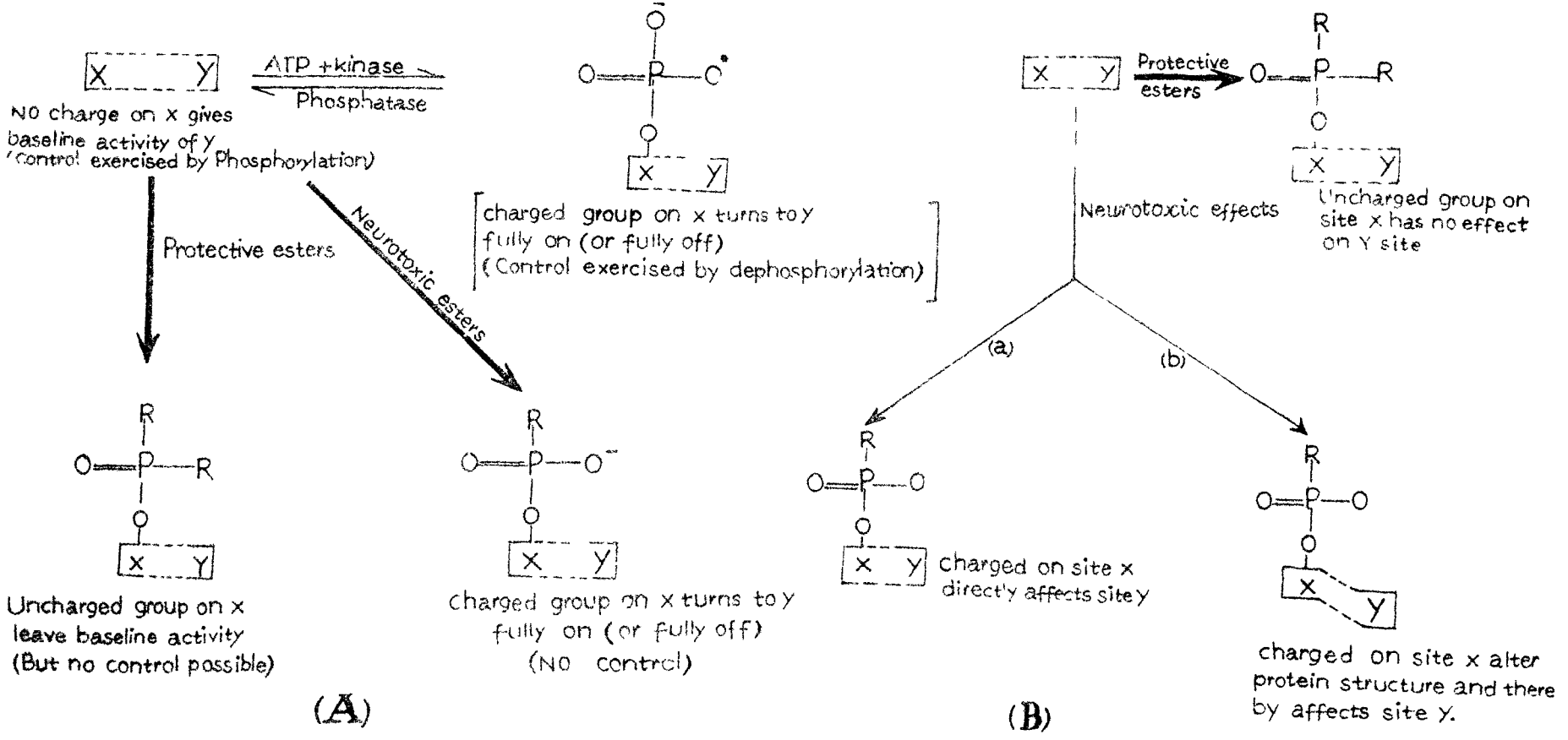
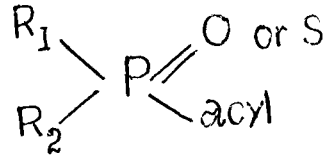


FIG. 2: Possible effects of substituents at the active site of neurotoxic esterase

1.7. Structure Activity Relationship:

The activity of an organophosphorus compound is highly dependent on its chemical structure. Slight structural changes greatly alter the whole spectrum of biological activity. In 1937, Schrader, proposed a formula (XVIII) for insecticidal organophosphates which has proved most fruitful in the synthesis of new compounds.



(XVIII)

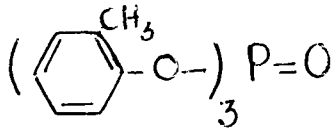
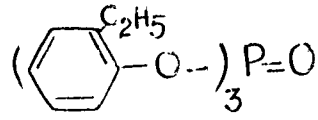
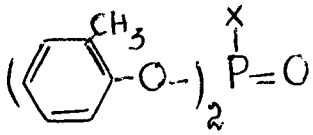
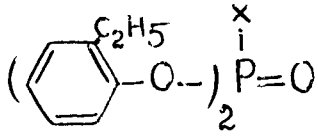
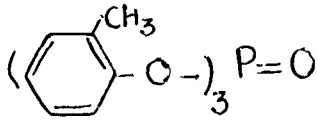
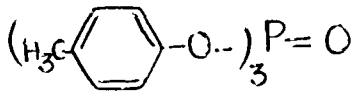
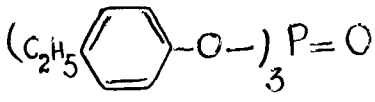
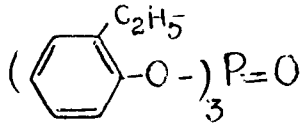
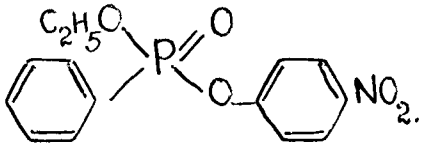
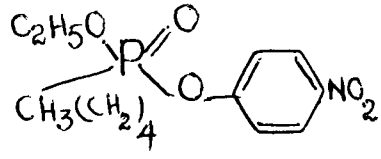
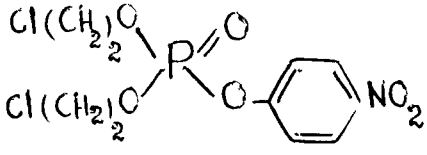
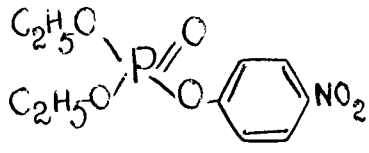
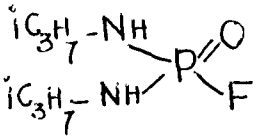
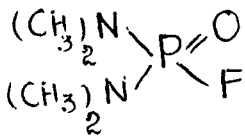
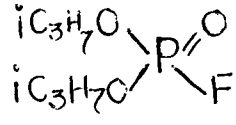
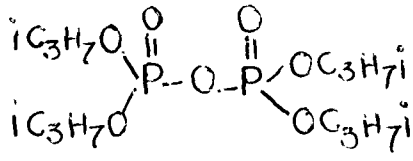
In this structure a pentavalent phosphorus with sulfur or oxygen is directly bonded and R_1, R_2 may be alkoxy, alkyl, aryloxy, aryl or amino groups; the acyl group can be extremely varied like pyrophosphate, fluoride, cyanide, substituted-phenoxy, thio-alcohols, aliphatic, heterocyclienol. After the substitution of these groups in Schrader's proposed formula, the activity of the compound will be changed according to the substitution of different groups. Clark et al., (1964) described phosphorylating compounds as P-XY: system in which the electron of the P-Y bond can be accepted by Z. X, Y and Z are usually N, C, H, O, S or halogen. Therefore, the phosphoryl acting potential of esters with a complex substitute can be fitted in this above system. The biological activity of organophosphates is to be considered as an inhibition of cholinesterase-splitting and other serine enzyme which, chemically, undergo phosphorylation of the serine-alcohol group at the esteratic site.

The inhibition of the enzyme depend upon the affinity of the inhibitor for the active center of the enzyme as well as upon the phosphorylation constants and therefore there is a direct correlation between inhibition and phosphorylating action. But the rate of phosphorylation is also dependent upon the different substitute. According to Ritter and Cutner (1961) the rate of formation of the non-reactivatable form (phosphorylation) in terms of half-life depends upon the alkyl groups of the phosphoryl radical and increases in the order; diethyl phosphate < dipropyl phosphate < dimethyl phosphate. Similarly Fukuto et al. (1961) described that the rate of enzyme inhibition was reduced if the compound had branching at C_1 and C_2 position in the alkyl group R, whereas the branching at C_3 and C_4 (e.g. R = iropentyl; isohexyl) raised the rate of inhibition. Therefore, when Ke (bimolecular rate constant of enzyme inhibition) was plotted against the log of toxicity, such a wide scattering of points resulted that no definite trend was perceptible (Fukuto et al., 1961). There are a number of examples showing that minor changes in structure or even the change of position of the same group leads to a change in the activity of the compounds as shown in Table - 2.

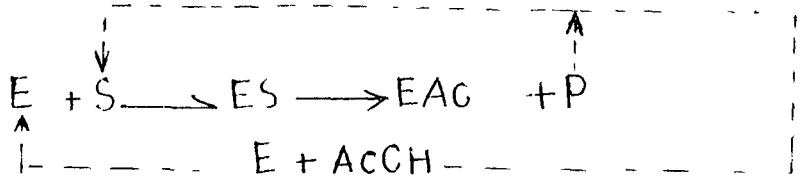
1.8. Organophosphates and Acetylcholinesterase:

Acetylcholinesterase is a enzyme which hydrolyzes choline ester faster than non-choline esters. Two classes of cholinesterase are recognized "True and Pseudo Cholinesterase". The true

Table - 2 : Structure and Neurotoxic Action of Different Organophosphorus Compounds.

Neurotoxic	Non-Neurotoxic	References
		Bondy et al., 1960 Aldridge & Barnes, 1961
		Bondy et al., 1960
		Aldridge & Barnes, 1961
		Aldridge & Barnes, 1961
		Aldridge & Barnes, 1966a
		Aldridge & Barnes, 1966a
		Barnes & Dens, 1953
		Davison, 1953

cholinesterase are found in nervous tissue as well as in red blood cells while pseudocholinesterase is found in plasma and other tissue of the body. The native molecule of acetylcholinesterase consists of four subunit of average molecular weight 6,400 ± 4,000 (Leuzinger et al., 1969). As far as the structure is concerned it consists of two non-identical chain. Thus structurally speaking, it is a dimer. The activity of this enzyme is blocked by organophosphates. Due to this inhibition the acetylcholine accumulates at the post synaptic membarane which is then unable to return to its original state. This accumulation of acetylcholine at the synapses causes the CNS poisoning (Engelhard et al., 1967). Krupka and Laider (1961) and Kurpka (1962), described the structure of the active site, kinetics of enzyme action and inhibition of the enzyme in detail. He explained all the active site of acetylcholinesterase and its mode of action with evidences. As shown in Scheme - 4, E is enzyme and S is the substrate. Both of them combine to form a substrate-enzyme complex ES. This complex is called michaelis complex. The ES complex splits the acetylcholine and ES gets changed to acetylated enzyme EAC. In the last stage, hydrolysis takes place, by which the enzyme get reformed and acetate together with choline returns to acetylcholine synthesis.



SCHEME -4

There are two active sites in this reaction. First is "anionic site" which binds the cationic part of the substrate by Coulomb forces. Second is the "esteratic site" having one alcohol group, together with the activating acid and basic groups. The distance between these two active sites is less than 4.5 Å (Fig.3). Basic groups of acetylcholinesterase are very much closer to imidazole ring of histidine molecules. With the help of a proton, one amidazole ring activates serine alcohol group for acetylation. At the active site imidazole ring reacts with water molecule and acetylserine gets hydrolysed.

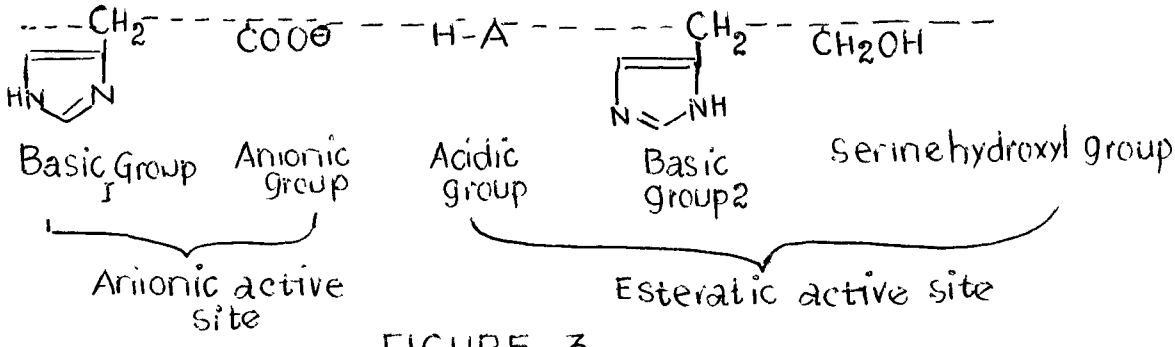


FIGURE -3

Later, in 1969 Leuzinger et al. also described the structure of acetylcholinesterase. According to them the acetylcholinesterase has four chains. This model is called Leuzinger four chain model of AChE. This is one of the important contributions in the theory of AChE. At present it is difficult to understand the mechanism of AChE inhibition by different inhibitors (e.g. interaction by Coulomb's forces, by hydrophobic interaction etc.). AChE and BuChE possess different properties due to the difference in their hydrophobic regions. For this difference in AChE and BuChE, Russian authors Kabachnik et al. (1970) gave a very interesting hypothesis. Because the interaction between substrates and anionic centres in AChE is governed by Coulomb forces, and in BuChE additionally by vander waals forces, the hydrophobic regions prevent a "paralitic"

sorption of acetylcholine by other active sites in the peptide chain. Acetylcholine contains a highly hydrophilic group which by sorption to other sites would retard the enzymatic hydrolysis of acetylcholine. The hydrophobic regions push this substrate to the active centre of AChE, thus ensuring maximum turnover. These workers also suggested that such lipophilic regions play the role of an "energy hill" from which the substrate "rolls down" to the active centre of acetylcholinesterase. A similar state may also be obtaining at the choline receptor site, i.e. sorption and desorption rates of the substrates are influenced by the size of the alkyl chains. In this way, the cholinomimetic effect of acetyl choline changes to a blocking effect (Kabachnik et al., 1970).

1.9. Organophosphate and Carbamates:

Organophosphates and carbamates are used as perticides/ insecticides and both of them are known as antichlinesterase. Carbamates have been known for longer time than the organophosphorus compounds. But the biochemical mechanism of intoxication is much better understood for the organophosphates than for carbamates (W.H.O. 16th report, 1967). Structurally, there is a difference of only phosphorus moiety. In carbamates, there is no phosphorus atom, otherwise both compound possess the aliphatic as well as aromatic structures. Organophosphate may itself bring about the AChE inhibition directly or through some compound first metabolized in the body and converted into an inhibitor. The rate of reactivation depends on the chemical structure of the insecticide. Similarly, in carbamates direct inhibition of AChE has been reported.

The available evidence suggests that the mechanism is analogous to the phosphorylation observed with organophosphates, called carbamoylation of the active centre of the enzyme. The unmetabolised compounds being capable of inhibiting the enzyme. The spontaneous reactivation of carbamoylated enzyme is more rapid than that of the phosphorylated enzymes. Carbamates not only undergo carbamoylation, but decarbamoylation of inhibited enzyme also takes place. In organophosphates spontaneous reactivation is not the only reaction that the inhibited enzyme may undergo. It may also be transformed into a state where no spontaneous reactivation occurs and where oximes are no longer capable of reactivating it. This phenomenon is called "Aging" and is characterized by removal of one of the alkyl groups from the phosphoryl group attached to the enzyme. In carbamates no "aging" phenomenon has, as yet, been observed (16th W.H.O. report, 1967), particularly with monomethylcarbamoylated enzyme, either in 'in vitro' or 'in vivo'. Recently, it has been shown that in carbamates, there is a greater ratio between the median dose producing symptoms (ED_{50}) and the LD_{50}, as compared with organophosphates. This is consistent with differences in the kinetic data on the action of these two classes of compounds on cholinesterase. Therefore, in carbamates the early stage inhibition is rapid and it is difficult to produce a severe degree of inhibition in these cases, because the rate of reactivation approaches that of inactivation. These differences in behaviour regarding enzyme inhibitors are important when we compare the toxic effects of carbamates and organophosphates.

1.10. Effects of organophorphate on amino acid neurotransmitters:

It has been suggested that amino acids play an important role in the normal function of the central nervous system and may also be transmitters at certain central synapses (Phillis, 1970). Recently, Nar and Nateen (1974) have reported DDT-induced lowering of the amino acid content of the mice brain. Furthermore, Papia et al. (1967) observed significant diminuition of free amino acids in brain of mice during drug-induced convulsions. The excitatory and depressant action of some amino acids when iontophoretically applied to central nervous system neurones resulted in investigations into their possible role as neurotransmitters (Curtis et al., 1965; Curtis, 1974). Evidence for such a function is based on information regarding the synthesis and storage of amino acids, the release from presynaptic terminals, the interaction with postsynaptic receptors which results in conductance changes similar to those induced by synaptically released transmitters, the parallel effects of antagonists of amino acids and transmitter actions, and the processes responsible for the inactivation of amino acids in the synaptic environment (Curtis, 1974). Glycine and gamma aminobutyric acid are known to be inhibitory transmitters. The evidence for aspartate and glutamate as excitatory transmitter is based on their distribution in the brain and spinal cord and the excitation by both amino acids of central neurones. Other amino acids may also be important as transmitters including alpha and beta-alanine, cysteate, cystathionine, cysteine sulphate, hypotaurine, taurine, proline and serine (Curtis, 1974). Recently Grezerstuk et al. (1974) have investigated the effect

of some pesticides on the content of protein and free amino acids in potato tubers. Information on the effect of organophosphate insecticides on free amino acid content of the different regions of the mammalian brain is still lacking. In view of the recent studies dealing with the regional variation in amino acid content of the rat central nervous system (Kandera et al., 1968; Cutler and Dudzinski, 1974), it would be of particular interest to investigate the effects of organophosphate pesticide dichlorvos-induced changes in putative neurotransmitter amino acids content of discrete regions of the rat brain and spinal cord.

1.11. Effects of organophosphates on monoamines:

It is well established, however, that monoamines (catecholamines and 5-HT) are present in the brain tissue, where they occure, atleast in part, in nerve fibres and nerve terminals. Monoamines are unevenly distributed in the brain, mainly in those regions to which autonomic, or vegetative, functions are attributed (Pfeiffer, 1966). Murar and Kand (1965) have reported that organochlorine pesticide-dichlorvos, similar in action to the other organophosphate pesticides, inhibits cholinesterase and that the accumulating acetylcholine stimulates the release of catecholamine. Furthermore, Carlsson (1974) has shown that physostigmine causes a decrease in brain dopamine and norepinephrine and accelerates the disappearance of brain catecholamines after inhibition of tyrosine hydroxylase. Interestingly, Fleur and Van Meeter (1977) have reported that an infiltration of parathion, a sulphur - containing organophosphate, alters turnover and levels of endogenous dopamine and norepinephrine.

The changes vary in different areas of rat brain. The brain is a heterogeneous tissue both anatomically and histologically. This heterogeneity is of great importance in the evaluation and interpretation of biochemical findings (Hertz, 1969). In experimental organochlorine pesticide - dichlorvos toxicity, so far there are no reports on the levels of dopamine, norepinephrine and 5-hydroxytryptamine (5-HT) in the different regions of the rat brain. It is, therefore, appropriate to estimate the concentration of catecholamines and 5-HT in the cerebral hemisphere, cerebellum, brain stem and spinal cord in an organophosphate pesticide-dichlorvos induced toxicosis in rats.

1.12. Effects of organophosphate on lipid peroxidation:

Lipids are essential components of all cellular structure in the brain. The total amount of lipids in both grey and white matter are large, although the lipid content of white matter (approximately 65% by unit dry weight) is larger than that of grey matter (approximately 35-40%) (Friede, 1966). Lipid peroxidation is the reaction of oxidative deterioration of polyunsaturated lipids. Peroxidation involves the direct reaction of oxygen and lipid to form free radical intermediates and to produce semistable peroxides (Tappel, 1970). It is generally believed that lipid peroxidation is a process involving the lipid of biological membrane (Plaa and Witschi, 1976). Kehrer and Autor (1977) described the changes in the fatty acid composition of rat lung lipids during development and following age-dependent lipid-peroxidation and was suggested that the age-dependent lipid-peroxidation is probably not a membrane associated

phenomenon (Kehrer and Autor, 1977). There are two kinds of lipid-peroxidation in tissues, one spontaneous and non-enzymatic (Hochstein and Ernster, 1963) and the other catalysed by Nicotinic acid Adenine dinucleotide (reduced) and localised in the microsomal part of the cell (Slater and Sawyer, 1971). Although direct uptake of oxygen will give a true nature of lipid-peroxidation, the more commonly employed technique is by estimating the production of malonaldialdehyde (one of the end products of fat peroxidation) by the pink pigment formed with 2-thiobarbituric acid (Kartha and Krishnamurthy, 1978). It has been reported that tissue most susceptible to lipid-peroxidation appear to be those with low mitotic rate such as brain (Barber and Wilbur, 1959). Recently, Kartha and Krishnamurthy (1978) reported that the different tissues from normal rat, the brain showed a considerably high degree of peroxidation, while liver, kidney, spleen and heart homogenates showed comparatively low peroxidation. Because of its wide-spread toxic effect, lipid-peroxidation might induce pathological lesions, as has been observed in the case of toxicity induced by iron (Goldberg et al., 1962) and CCl_4 (Slater, 1972). It has been suggested that lipid peroxidation may be the key event in the pathology of CCl_4 - induced hepatotoxicity (Recknagel, 1967). Furthermore, Kitada et al. (1977) reported that the addition of EDTA to the incubation mixture containing rabbit liver microsomes and ferrous ion resulted in a 2-fold increase of lipid-peroxidation activity. Such an enhancement was not observed in rat liver microsomes. Therefore, it is likely that lipid-peroxidation activity in rabbit liver microsomes may account for the insufficiency of an EDTA-like factor(s) in rabbit liver microsomes

(Kitada et al., 1977). Interestingly, Rao and Pandya (1977) described that liver lipid peroxidation was increased in the rat after the intraperitoneal administration of benzene, ionex, petrolium ether and gasoline. To my knowledge, the effects of organophosphate-pesticides on lipid-peroxidation are limited and particularly, the effect of organophosphate-dichlorvos on lipid peroxidation is not known, therefore, it would be of particular interest to investigate the rate of lipid-peroxidation in different regions of the rat brain after the administration of three doses of organophosphate dichlorvos.

1.13. Light and electron microscopy in organophosphate poisoning:

A survey of the available literature revealed that some organophosphates produce delayed neurotoxic effects - ataxia, weakness of limbs, muscle twitching and rigidity in man (Hidetrip et al., 1953), rat (Majno and Karnovsky, 1961) and other animals (Aldridge et al., 1969). These effects have been attributed to changes in peripheral nerves, degeneration or demyelination (Cavanagh, 1954). The likehood of TOCP (organophosphate) coming in contact with the skin in industrial workers and farmers is great as it is contain in some plasticizers, lubricant, petrol additives, lacquers and pesticides. That skin absorption is possible was demonstrated by Glees and White (1961). They showed nerve fibres degenerations in the anterior medial tract of the cord of hen after painting 0.1 ml TOCP/kg on the comb. Motor neurons did not show significant changes but silver preparations demonstrated characteristics terminal degenerations, fragmentation and granulations of axones. Long tract and axodendritic synapses were

affected. Oral administration of Cortirone acetate (25 mg daily prevented or alliviated neurological and functional disorders caused by TOCP (Cleer, 1961). Hereford and Cleer (1963) described ascending degenerations in the spinocerebral tract to its termination in the anterior and the posterior lobes of the cerebrum. Preterminal degeneration was found in the nucleus of descending route of the trigeminal and in the lateral reticular nucleus of the medulla. The ascending degeneration was more dark and occured slightly before the descending. The early ataxia was attributed to the damage to the ascending system, the supervening paralysis to that in the descending ventral tract (Hereford and Cleer, 1963). Cleer and Jamnik (1965) and Jamnik and Cleer (1966) further reported that chemically (TOCP) induced fibre degeneration in the central nervous system and chromatolysing spinal neurons in the chick, following tri-orthocresyl phosphate intoxication. On the other hand, Ahmad and Cleer (1971) observed large number of laminated cytoplasmic inclusion bodies in the spinal cord of hen intoxicated with tri-ortho-cresyl phosphate. They postulated that phospholipids become unmasked during a degenerative process and arrange themselves as alternating dark and less dark layers of the so-called laminated bodies. Additionally, Vij and Kanagaruntheram (1972) showed the damage caused by TOCP intoxication on the peripheral nerve endings of slow loris. Krishnamurti et al. (1972) have shown electron microscopic evidence of toxic effects of tri-ortho-cresyl phosphate on digital Pacinian corpuscles of slow loris. Extensive degeneration of the myelin lamellae in the proximal segment of the nerve fibre was detected. Evidences of mitochondrial degeneration, in the form of swollen profiles and loss of cristae, together with neurofilamentour accumulation were also reported by Krishnamurti et al.,

(1972). Increased in the number of cytoplasmic filaments and a hypertrophy of endoplasmic reticulum were observed by Le Vay et al. (1971) in an ultrastructural investigation on neurons of spinal ganglia in DFP poisoned hens. Additionally, mitochondrial alteration in ganglia cells of a prosimian primate were reported by Ahmad and Gleer (1976). It is interesting to note that similar changes were not detected in neurons of avian spinal ganglia by Le Vay et al. (1971). However, recently Spoerri and Gleer (1979) have clearly demonstrated enlarge and swollen mitochondria with complete to partial disruption of cristae and loss of matrix after 10 days injection in spinal ganglia of Gallus domesticus. Several of the degenerating mitochondrial profiles became osmophilic. Lipofuscin granules of various sizes and shapes were also associated with the altered mitochondria. Their internal structure were heterogenous, showing dark granules, laminations and vacuoles. Interestingly, in some cells the mitochondria were markedly dense and elongated while in other early stages of degeneration were visible. The cisternae of the endoplasmic reticulum were relatively short and dialated (Spoerri and Gleer, 1979). To my knowledge, the neuropathological studies of cases with organophosphate-pesticide dichlorvos intoxication are limited and, electron microscopy has, so far, not been utilized to investigate the effects on the hypothalamus, hippocampus, cerebellum and spinal cord. Therefore, it would be of paramount importance to investigate the histochemical and ultrastructural changes in the rat brain, following subacute organophosphate - dichlorvos intoxication.

1.14. Psychological behaviour in organophorphates poisoning:

Most of the psychological behavioural studies have been done in man particularly industrial and agricultural workers acutely exposed to a variety of perticides. In the early 1950's a group of scientists at the university of Colorado Medical center, investigated certain psychological, neurological, clinical-neurophysiological and a number of biochemical parameters after the acute confusional states induced by organophorphates exposures in human beings (Metcalf and Holmes, 1969). Neurological examinations revealed multiple minor signs such as generalised weakness and confusion shortly after exposure. The central effects of organophosphates exposure are casually related to specific impact of organophorphate compounds on deep midbrain, acetylcholinesterase-rich pontine centers (Metcalf and Holmes, 1969). It is also known that control of sleep-wake fulness cycle is partially dependent upon cholinesterase - acetylcholinesterase mechanism (Hernandez-Peon, 1965); that specific sleep stages are under the influence of the mid and rostral pons (Jouvet, 1965); and that these structures are partially responsible for modulation of transmitted sensory input to the cortex.

In experimental animals a number of reports have been published on open field behaviour, motor activity, EEG after the administration of psychoactive drugs, alcohol and other sedative (Irving et al., 1969; Taylor and Synder, 1971; Thornburg and Moore, 1972; Jenkert et al., 1973). A connection between stereotyped behaviour induced by DOPA in various experimental animals by

increasing the concentration of dopamine in the specific centres of extrapyramidal system is assured (Scheel Kruger and Randrup, 1967). It has been reported that a variety of CNS active drugs modify the open field behaviour in rats on acute administration. Depending upon the site and mechanism of action the various open field parameters e.g. ambulation, rearing, preening and defecation show varying responses. Ambulation is described as horizontal (simple) stereotypy (Dandiya et al., 1969). It is indicative of intact muscular co-ordination. Norepinephrine is said to be main modulator of simple stereotypy in open field test (Kulkarni and Dandiya, 1972). Rearing is a vertical (complex) stereotypy (Gupta et al., 1972). It is said to be an indication of cortical stimulation (Lal, 1965). It is independent of horizontal stereotypy. Brain dopamine levels have an important role in controlling complex stereotypy. -Dopa in the presence of RO-4602 markedly increased rearing response. Preening is a behavioural response which is said to decrease with drugs causing stimulation of ambulation and rearing and vice versa. It can also be inhibited by reducing cortical inhibition. Defecation is an emotional response due to sympathetic nervous system over-activity (Gupta et al., 1971). It can also be caused by excitatory effect produced on the nervous loci of posterior hypothalamus which controls the activity of the sympathetic nervous system. Motor activity is the hyperactivity of the animals and norepinephrine level is responsible for this behaviour (Matussek and Ruther, 1965). 5-HT and other catecholamine metabolite have also been accounted for increased motor activity (Scheckel et al., 1969). In the light of the above mentioned findings, it would be appro-

priate to include the behavioural effects of dichlorvos as one of the tasks of the present study.

1.15. Diagnosis, sign and symptoms of organophosphorus compounds:

Diagnosis of organophosphorus compounds depends upon the type of insecticide, whether, it is direct inhibitor or indirect inhibitor of acetylcholinesterase. In case of direct inhibitor the sign and symptom appear atonce while in case of indirect inhibitor, the symptoms may not appear untill after the individual has left work. In some instance illness may develop during the night, and its association with occupational poisoning may not be recognized immediately. Organophosphates poisoning were not found in central nervous system alone but it has reported that nearly every part of the body shows the signs and symptoms of poisoning (Holmstedt, 1959). Diagnosis can also be made from a history of exposure followed by the onset of all or some of the signs and symptoms listed in Table 3 & 4.

1.16. Therapy of organophosphates poisoning:

Serious poisoning by organophosphates may follow ingestion inhalation, skin contact and is likely to occur in cases of occupational exposure in public health programmes. Klimmer (1964) gave the practical direction for the first aid treatment of organophosphate poisoning.

Table - 3 : Signs and symptoms of organophosphate poisoning following local exposure.

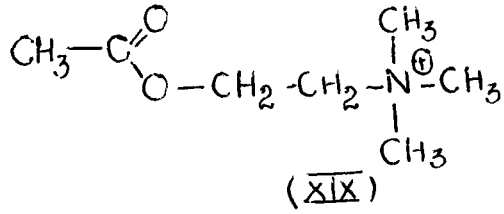
Site of action	Signs and symptoms
Pupils	Miosis, marked, usually maximal (pin-point), some times unequal.
Ciliary body	Frontal headache, eye pain on focusing, slight dimness of vision, occasional nausea and vomiting.
Conjunctivae	Hyperaemia.
Nasal mucous membrane	Rhinorrhoea, hyperaemia.
Bronchial tree	Tightness in chest, some times with prolonged wheezing expiration suggestive of bronchoconstriction or increased secretion, cough.
Sweat glands	Sweating at site of exposure to the liquid.
Striated muscle	Fasciculations at site of exposure to the liquid.

Table - 4 : Signs and symptoms of organophosphate poisoning following systemic absorption.

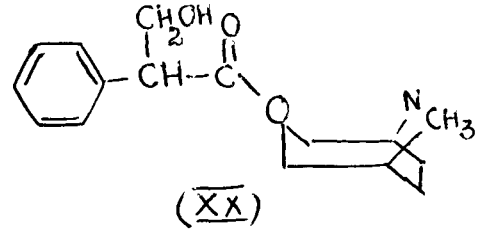
Site of action	Signs and symptoms
Pupils	Slight miosis (occasionally unequal), later more marked.
Ciliary body	Burning of vision.
Bronchial tree	Tightness in chest, with prolonged wheezing expiration suggestive of bronchoconstriction or increased secretion, dyspnoea, slight pain in chest, increased bronchial secretion, cough.
Gastro-intestinal system.	Anorexia, nausea, vomiting, abdominal cramps, epigastric and substernal tightness with "heartburn" and eructation, diarrhoea, tenesmus, involuntary defecation.
Sweat glands	Increased sweating.
Salivary glands	Increased salivation.
Lachrymal glands	Increased lachrymation.
Heart	Slight bradycardia.
Bladder	Frequent or involuntary micturition.
Striated muscle	Easy fatigue, mild weakness, muscular twitching, fasciculations, cramps, generalized weakness including muscles of respiration, with dyspnoea and cyanosis.
Sympathetic ganglia	Pallor, occasional elevation of blood pressure.
Central nervous system.	Giddiness; tension; anxiety; jitteriness; restlessness; emotional lability; excessive dreaming; insomnia; nightmares; headache; tremor; apathy; withdrawal and depression; bursts of slow waves of elevated voltage in EEG especially on overventilation; drowsiness; difficulty in concentrating; slowness of recall; confusion; slurred speech; ataxia; coma with absence of reflexes; cheyne-stoker respiration; convulsion; depression of respiratory and circulatory centres with dyspnoea; cyanosis and fall in blood pressure.

Later in 1967, W.H.O. 16th report published the treatment of anticholinesterase poisoning. According to the report, in addition to the use of drugs - removal of the toxic agent and decontamination of exposed skin with an alkaline solution of soap and water were recommended. Drugs used for the treatment of organophosphate poisoning are classified under the following two headings:

1.16.1. <u>Atropine</u>: Atropine sulfate is recommended as the first aid in the poisoning by organophosphorus compounds.



(XIX)



(XX)

The structural similarity of atropine (XX) to its natural agonist acetylcholine (XIX) is very apparent. Strictly speaking, atropine is not a direct antagonist of organic phosphorus compound but rather a competitive reversible inhibitor of acetylcholine at the receptor sites (Bert and Schmidt, 1973). Therefore, atropine attacks at the receptor and has proved the most effective agent for the treatment of organophosphate poisoning. It should be injected immediately after the appearance of any of the serious signs of anticholinesterase poisoning. Klimmer (1964), recommended that in cases of severe poisoning,

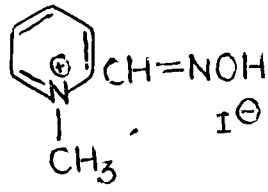
3-5 mg atropine be injected (i.v.) as an initial dose. Thereafter a further 2.0 mg of atropine be administered every 10-15 minutes until there is a definite improvement. According to the 16th W.H.O. report in 1967 the dose of atropine may be adjusted as the symptoms appear.

1) Mild symptoms	1-2 mg atropine (i.v.)	30 min. interval
2) Moderately severe.	2-4 mg " "	10 min. interval
3) Severe	4-6 mg " " (but repeated dose 2.0 mg)	5-10 min. interval

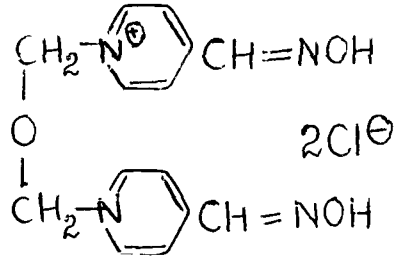
Any patient sick enough to receive even one dose of atropine should be under medical observation for atleast 24 hrs, because the symptoms may reappear, especially in organophosphate poisoning.

1.16.2. <u>Oxime - reactivators</u>: During the recent years it has been demonstrated that atropine is not the only effective drug used for organophosphate poisoning. But certain oximes when injected with atropine in cases of organophosphate poisoning show better result than atropine alone. Oximes reactivate phosphorylated cholinesterase and act at the site to correct the biochemical defect rather than merely relieve symptoms (W.H.O. 16th report, 1967). Pralidoxime iodide (XXI) (2-formyl-1-methyl pyridinium iodide/2-pyridinium aldoxime methiodide/2-PAM) has been used for accidental poisoning by organophosphates. This oxime is injected in

dose of 100-300 mg/minute (i.v.) upto the 2000 mg at one time. This dose produces no untoward signs or symptoms and no change in blood pressure or cardiac rate. In case of administration of pralidoxime upto the repeated dose totalling 40 gm by i.v. no side effects were noted and recovery was hastened.



(XXI)

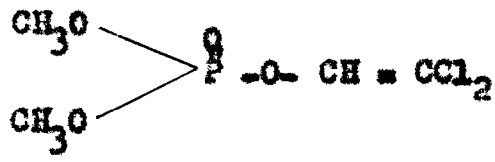


(XXII)

Toxogonin (XXIII) (1,1'-(oxydimethylene) bis (4-formyl-pyridinium chloride), dioxime) is another reactivator which is used for organophosphate poisoning. The reactivating potential of toxogonin is higher than pralidoxime but its toxicity is also slightly higher. Toxogonin is administered intravenously in dose of 250-500 mg in adult. However, the safety in man of toxogonin has not yet been established (W.H.O. 16th report, 1967).

1.17. Dichlorvos - an organophosphate:

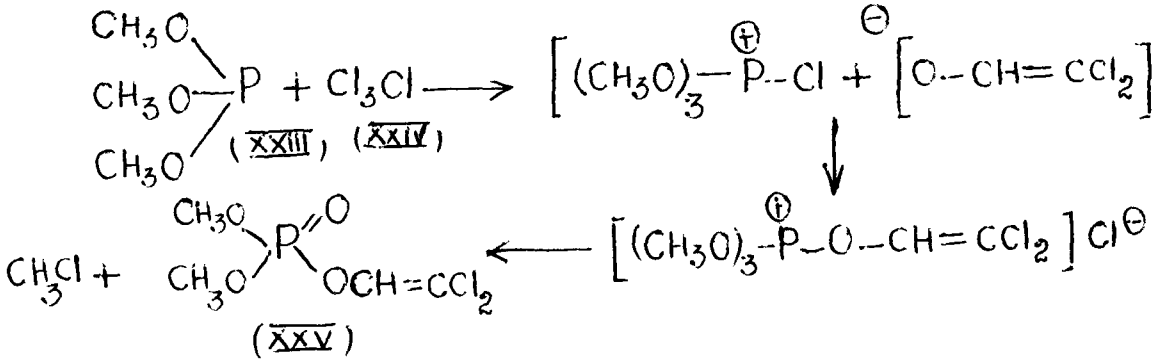
Dichlorvos (DDVP) is an organophosphorus compound having the following physico-chemical properties:

Common name	Dichlorvos, DDVP
Market name	Nuvan, Roqos, vapone
Manufacturer	Ciba Geigy India Ltd.
Chemical name	0,0-dimethyl-2,2-dichlorovinyl phorphate.
Structural formula	
Empirical formula	$C_4H_7Cl_2O_4P$
Molecular weight	220.98
Specific gravity	1.420 at 15°C
Purity (Technical grade)	93.6% (determined by iodometric method).
By product	> 7%
Solubility	1% in water, soluble in most organic solvents.
Colour (Technical grade).	Yellowish to colourless.
Stability (Pure)	Hydrolyses slowly in neutral and acid solutions, but rapidly in alkaline medium.
Stability (Technical grade)	Dichlorvos is stable when stored in glass and certain plastic materials like polyethylene.
Boiling point	35°C at 0.05 mm Hg. 53°C at 0.2 mm Hg.
Flash point	> 100°C.
Vapour pressure	$1.2.10^{2}$ mm Hg at 20°C $3.0.10^{-2}$ mm Hg at 30°C $7.0.10^{-2}$ mm Hg at 4°C

Volatility	145 mg/m^3 at 20°C 350 mg/m^3 at 30°C 800 mg/m^3 at 40°C
Corrosiveness	Dichlorvos corrodes mild steel, in the absence of moisture it does not corrode aluminium, nickel, Hastelloy B or stainless steel.

1.18. Synthesis of Dichlorvos:

Dichlorvos was synthesized by various groups in the early fifties. Perkow obtained it (XXV) in 1955 through the reaction of trimethylphosphite (XXIII) with chloral (XXIV), the reaction proceeded as shown with the elimination of methyl chloride (Scheme - 6).

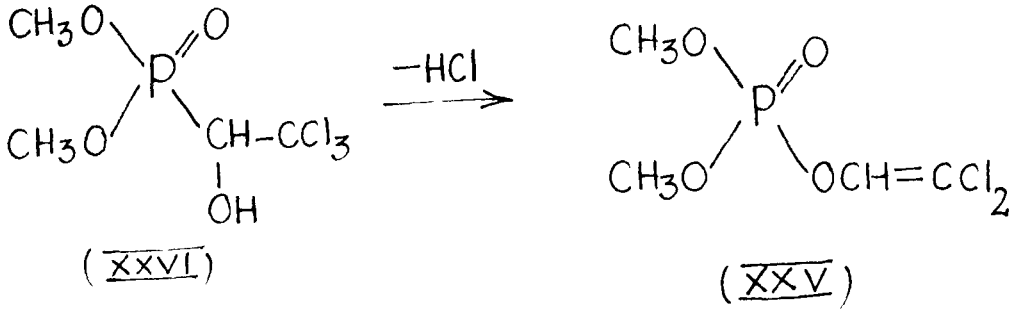


SCHEME-6

The reaction is exothermic and can be carried out with or without solvent like benzene or toluene at room temperature, at higher temperature and at normal or reduced pressure. At the end of the reaction the end product can be purified by fractional distillation.

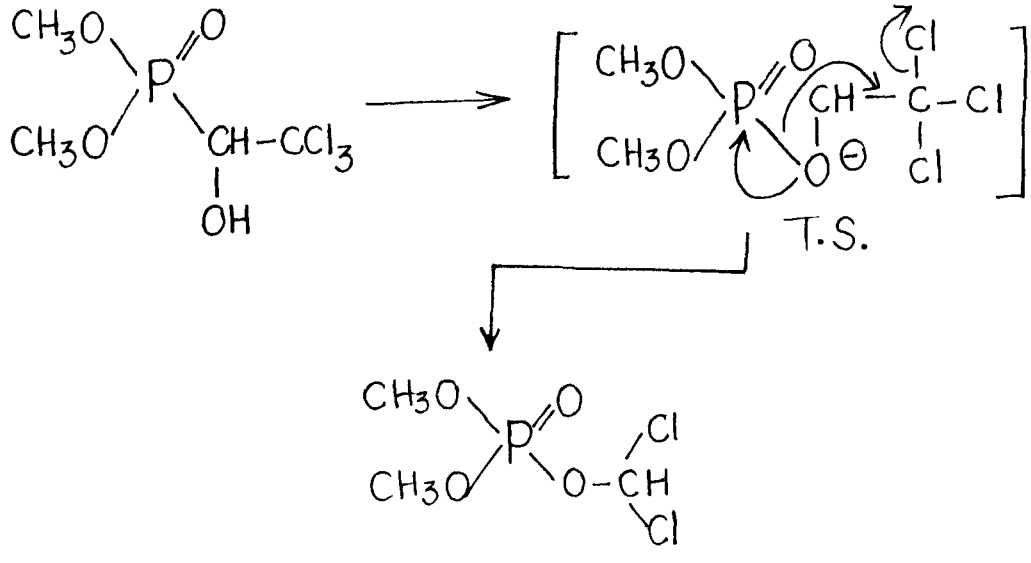
Another approach to the synthesis of dichlorvos (XXV) was worked out by Mattson et al. (1955). Trichlorfon (XXVI) obtained

from dialkyl-phosphite and choral is subjected to hydrolysis under baric condition.



(XXVI) (XXV)

This leads to the elimination of hydrochloric acid and formation of dichlorvos through a rearrangement for which the following mechanism have been purposed.



Some other methods have also been developed for the manufacture of this important pesticides.

1.19. Toxicity of Dichlorvos:

The results obtained by different investigators are summarized in Table 5,6 and 7.

1.20. Analysis of Dichlorvos:

Several methods of analysis have been developed for dichlorvos, which are as follows:

1. Iodometric method
2. Colorimetric method
3. Mercaptometric method
4. Infrared spectroscopy
5. Gas chromatography

1. Iodometric Method: This method is used in the analysis of technical material and formulations. Dichlorvos consumes iodine in a strongly alkaline solution, but not in sodium carbonate, while by products in technical dichlorvos consume iodine equally in a strongly alkaline solution and in sodium carbonate. Therefore, in this method it is

Table - 5: Acute Toxic dose of Dichlorvos (0,0-Dimethyl 2,2-dichlorovinyl phosphate).

Species	Effective dose concentration.	Effect	References
Mice (Male)	4 mg/kg (oral)	LD_{50}	Tracy (1960)
Mice*	124 mg/kg (oral)	LD_{50}	Yamashita (1960)
Rat (Male)	70-80 mg/kg (oral)	LD_{50}	Tracy (1960)
Rat (Male)	80 mg/kg (oral)	LD_{50} (i) (ii) (iii)	Durham et al.(1957) Gaines (1960) Carida (1962)
Rat (Female)	56 mg/kg (oral)	LD_{50}	
Rat (Female)	50-60 mg/kg (oral)	LD-50	Tracy (1960)
Rat*	900 mg/kg (Percutaneous)	LD-50	Klotzsch (1956)
Rat*	170 mg/kg (Percutaneous)	LD-50	Durham (1957)
Rat*	6 mg/kg (intraperitoneally)	LD-50	Arthur and Carida (1957)
Birds*(Mallards)	7.0 mg/kg (oral)	LD-50	Tucker and Crabtree (1970)
Leghorn hen (adult)	22.8 ± 1.6 mg/kg	LD-50	Kettering Laboratory report (1964)
Birds (Pheasants)	11.3 mg/kg (oral)	LD-50	Tucker and Crabtree (1970)
Dog*	100-316 mg/kg (oral) (Capsule)	LD-50	Hazleton Laboratories Inc.(1960).
Dog (Male)	11 mg/kg (oral)	Cholinesterase inhibition.	Snow and Watson (1973).
Dog (Female)	11 mg/kg (oral)	Severe toxicity, cholinesterase inhibition.	Snow and Watson (1973).
Dog (Male)	22 mg/kg	Moderate toxicity, cholinesterase inhibition.	Snow and Watson (1973).

Contd. on next page.

Dog (Female)	22 mg/kg	Fatal toxicity, markedly inhibition of ChE.	Know and Natron (1973)
Calf (2 days old)	10 mg/kg (oral)	Mark inhibition of cholinesterase.	Lawrence (1965)
Cow (Pregnant)	27 mg/kg (oral)	Clinical nontoxic.	Tracy (1960)
Horse*	50 mg/kg (oral)	Cholinesterase inhibition.	Jackson et al. (1960).
Horse*	10 mg/kg (oral)	No clinical sign of poisoning.	Younger (1965)

* Sex not stated.

Table - 6: Chronic Toxic dose of Dichlorvos (0,0-Dimethyl 2,2-dichlorovinyl phosphate).

Species	Effective dose or concentration.	Effect	References
Rat (Male)	10-20 mg/kg (oral)	50% cholinesterase inhibition.	Tracy et al. (1960).
Rat (Female)	30-40 mg/kg (oral)	Shock symptoms and died within 24 hrs.	Tracy et al. (1960).
Cattle	11 gm (in fodder)	No cholinesterase inhibition.	Tracy et al. (1960).
Cattle	30 gm (in folder)	Marked cholinesterase inhibition.	Tracy et al. (1960).
Cattle	155 gm (in capsule)	Marked cholinesterase inhibition	Tracy et al. (1960).
Cattle	169 gm (in capsule)	Shock symptoms recovery within 3 hours.	Tracy et al. (1960).

Table - 7 : Inhaled toxic dose of Dichlorvos (0,0-Dimethyl 2;2-dichlorovinyl phosphate).

Species	Effective dose or concentration	Effect	References
Rat	31-118 mg/m^3 of air	LD - 100	Durham et al. (1957)
Rat	0.41-1.13 mg/m^3 of air	ChE inhibition	Tracy et al. (1960)
Bird (Mallards)	75,000 ppm (for 5 days)	Toxic systems	Heath et al. (1970)
Fish (Blue Gills)	1 ppm (for 24 hours exposure)	Toxic systems	Cope (1965)
Rabbit	0.2-300 ug/lit. in vapour	No ChE inhibition	Durham et al.(1957)
Horse	0.2-300 ug/lit. in vapour	No ChE inhibition	i) Tracy et al. (1960) ii) Shell Development Co.(1962)
Horse	18 mg/m^3 of air (for 21 days)	Temporary ChE inhibition.	Tracy et al. (1960)
Monkey	7.5-17.9 mg/m^3 of air (for 2 hourr daily for 4 days)	Swift fall in ChE activity.	Hitter et al. (1961)
Monkey	0.1-0.5 mg/m^3 of air (for 50 days).	ChE inhibition	Durham et al. (1957)
Monkey	71 mg/m^3 of air (for 42 days)	ChE inhibition	Durham et al. (1959)
Monkey	6.9 mg/m^3 of air (30-60 minutes for one day)	No ChE inhibition	Durham et al. (1959)
Monkey	0.26-0.88 mg/m^3 of air (1 hour for 4 days).	No ChE inhibition	Hitter et al. (1961)
Monkey	0.7-1.0 mg/m^3 of air (8 hours for one day).	Slight inhibition of AchE.	Durham et al. (1959)
Monkey	1.3-1.4 mg/m^3 of air (8 hours	Slight inhibition of AchE.	Durham et al. (1959)

possible to determine the dichlorvos content by different titration. This method can also be used for the determination of dichlorvos in solution in hydrocarbons.

2. Colorimetric method: This method is based on the colour reaction of dichlorvos with resorcinol in sodium carbonate solution, after any impurities which might affect the reaction have been removed by the treatment with iodine. Therefore, by this method even small amounts of dichlorvos in air can be easily determined (Geiger and Furer, 1960; Buchler and Heinler, 1963; Buchler et al., 1965).

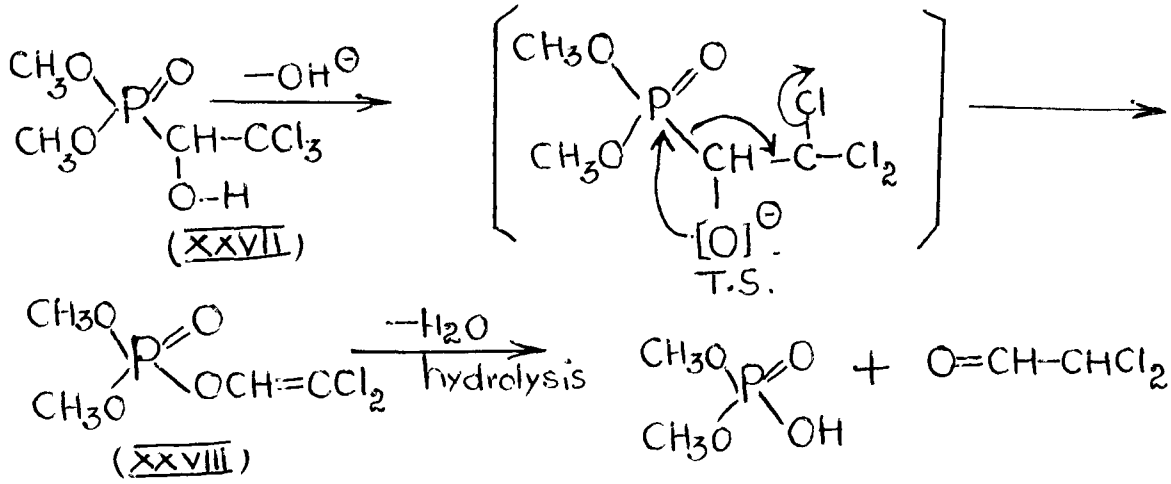
3. Mercaptometric method: This is the modified method of iodometric. In this case the dichlorvos formulations which, due to certain emulsifying additions, could no longer be analysed by the earlier iodometric method can be analysed. Mercaptans react with dichlorvos in alkaline solution and the rest of the mercaptans can be determined by iodometric back titration. Impurities which might affect the determination should already be removed by the sodium hydrogen sulphide extraction. This method is highly sensitive as compared with the preceding two methods.

4. Infrared spectrophotometry: Infrared spectrophotometry is very useful method for the determination of dichlorvos. In this method we determine the dichlorvos of the technical grade which is based on measurement of absorbance of the band at 10.2 u in methylene chloride solution.

5. Gas Chromatography (GLC): The gas layer chromatography is also known to be suitable for quantitative determination of dichlorvos. By this method we can determined even a very minute quantity of dichlorvos.

1.21. Chemical degradation of dichlorvos:

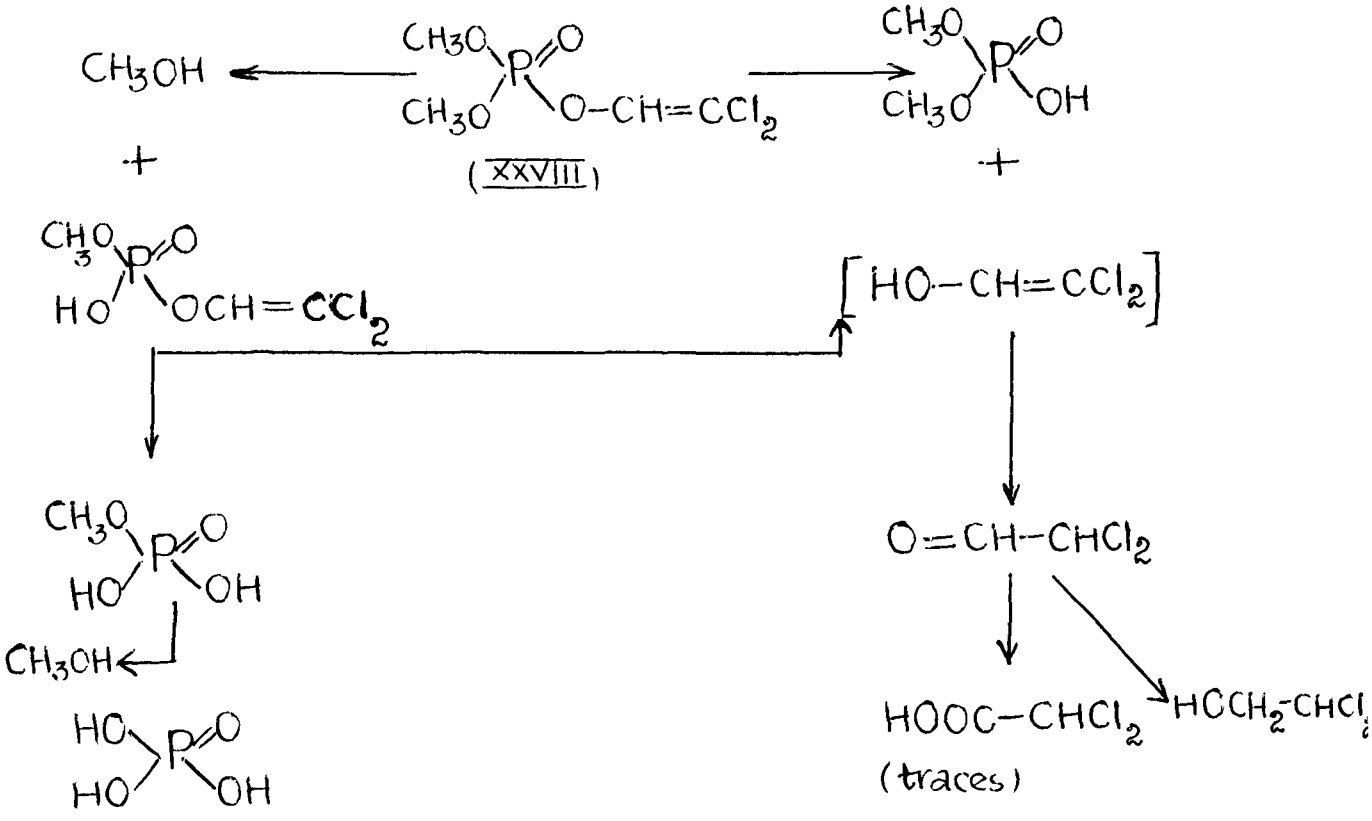
After the discovery of insecticidal properties of dichlorvos, Tracy in 1960 demonstrated the biological instability of dichlorvos 'in vivo' by a bioassay technique. Terry in 1960 showed that in acidic media, hydrolytic cleavage of the P-C bond and dealkylation was possible. In weakly alkaline medium, i.e. under physiological conditions, conversion of trichlorfon (XXVII) to dichlorvos (XXVIII) takes place as follows (Scheme - 9):



SCHEME-9

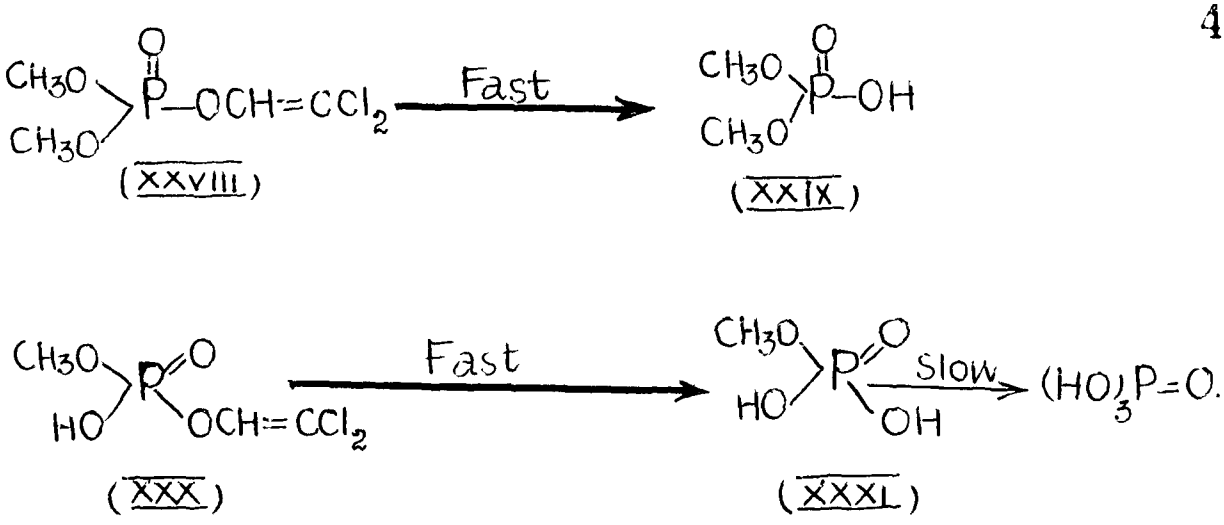
Following these preliminary studies, Casida et al. (1962) described the metabolism of dichlorvos in mammals. The C_1 fragments are eliminated in the form of unknown derivatives in the faeces and as CO_2 in the respiratory gases. Tests with naled in rats and cattle have shown that hydrolytic attack begins at the $-OCH_3$

grouping, whereby additional metabolites, such as dichlorobromoacetaldehyde are formed. Indeed this reacts readily with the sulfahydryl group (Casida et al., 1962). In the same year Hodgson and Casida described that in mammals dichlorvos (XXVIII) is presumably metabolized as shown in Scheme - 10.



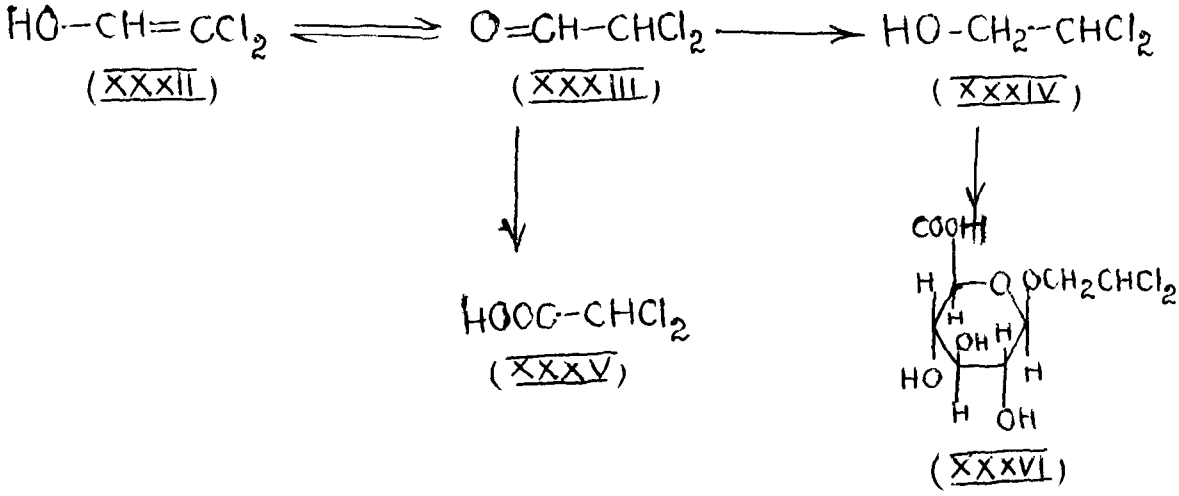
SCHEME -10

Later, in 1967 O'Brien described in detail the chemical degradation of dichlorvos in mammalian tissues. According to him the degradation of dichlorvos (XXVIII) was shown to occur by hydrolytic routes involving phosphatases, which are able to split the P-O-vinyl linkage and to a smaller extent the P-O-methyl bond (as shown in Scheme - 11 & 12). Whereas the dimethyl phosphate (XXIX) was not metabolized, but was rapidly excreted by the animal body, methyl phosphate (XXXI), presumably the product of hydrolysis of desmethyl dichlorvos (XXX), was slowly hydrolysed to phosphoric acid by a soluble enzyme of the rat liver.



SCHEME -II

In the animals tissue when dichlorvos or dermetnyl dichlorvos was hydrolysed it released a non-phosphorus moiety, which was subsequently degraded as follows (Scheme - 12).



SCHEME -12

Dichloroacetaldehyde (XXXIII) appeared to be formed by non-enzymatic transformation of the unstable dichlorovinyl alcohol (XXXII). Subsequently, reduction of dichloroacetaldehyde to

dichloroethanol (XXXIV) was accomplished by liver soluble preparations or purified alcohol dehydrogenase and required reduced nicotinamide aderine dinucleotide (NADH). Formation and excretion of dichloroethyl glucuronide (XXXVI) was consistent with an elimination pathway which was already demonstrated by Williams (1959). But the mechanism which led to the formation of small amount of dichloroacetic acid (XXXV) was not yet investigated (Ciba Monograph, 1971).

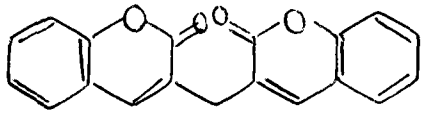
1.22. Mode of action of dichlorvos:

Dichlorvos is a highly effective contact, stomach and inhalation poison from the vinyl phosphate group of phosphoric ester compounds. It produces all of its known effects through direct inhibition of cholinesterase. As it is already described under "Chemical degradation of dichlorvos" No. 1.21, the end products would be phosphoric acid, carbon dioxide, water and traces of hydrochloric acid. Dichlorvos is absorbed from the gastrointestinal tract by way of the blood rather than the lymph (Lowe, 1966). Then it is rapidly detoxified by the liver (Gaines et al., 1966). This very rapid metabolism is reflected clinically in the prompt recovery of animals that survive poisoning. Therefore, in mammals, because of rapid hydrolysis, the compound formed by dichlorvos with cholinesterase is not stable while in case of insect, the hydrolysis of the dichlorvos-cholinesterase compound proceeds slowly. Insect one, therefore, not only destroyed by an acute dose but also by continual or regularly repeated applications of subacute doses of dichlorvos.

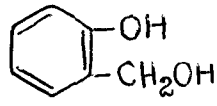
1.23. Coumarophorphate:

A large number of organophorphorus compoundr have been reparcd in the hope of finding r enhanced practical activities. Many of there involve replacement of rubstituents and introduction of fluorine in the molecule. During the courre of this rtudy it war felt that it mi ht be interesting to explore the behaviour of rome phorphorur derivative of dicoumarol.

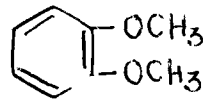
Dicoumarol (XXXVII), the dimer of 4-hydroxycoumarin with a methylene bridge, ar is well known, ir a powerful anticoagulant. It ir rtructurally rimilar to saliginine (XXXVIII) (O-hydroxy methyl phenol) which ir alro pharmacolo ically active like ar rine (XXXIX) a related type of coumarol.



(XXXVII)



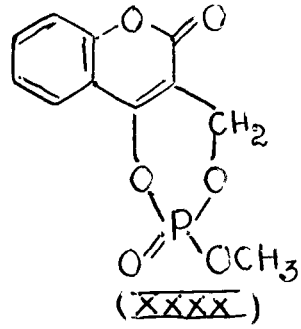
(XXXVIII)



(XXXIX)

4-Hydroxycoumarin reacts with formaldehyde under basic conditions to give 3-hydroxymethyl-4-hydroxycoumarin (XXXX) which reremblen raliginine in itr rubrtitution. This ir however, inrolible under the reaction conditions and condenres with an other mole of 4-hydroxycoumarin to ive dicoumarol. It appeared porrible that the reaction of 4-hydroxycoumarin with formaldehyde in prerence of phorphoric a id mi ht rtabilise 3-hydroxy methyl-4-hydroxy coumarin

(XXXX) as the phosphate ester which could then be used for pharmacological work. This turned out to be the case and the compound was prepared in this way. Unfortunately its lack of solubility in water did not allow further pharmacological tests. The work, is however, being pursued under a separate programme.



(XXXX)

1.24. Specific objectives of this study:

The present study was undertaken with the following main aims:

(i) To prepare organophosphorus compounds having a simple structure, more active for pests and insecticides but less toxic for mammals.

(ii) Behavioural changes in rats - open field behaviour (ambulation, rearing, preening and defecation) and locomotor activity after the administration of organophosphate-dichlorvos.

(iii) Quantitative estimation of acetylcholinesterase activity in different regions of the rat brain and spinal cord in organophosphate-pesticide-dichlorvos intoxication.

(iv) Quantitative estimation of levels of dopamine, norepinephrine and 5-hydroxytryptamine in different regions of the rat brain after the administration of organophosphate pesticide-dichlorvos.

(v) Quantitative estimation of the effects of organophosphate-dichlorvos intoxication on the concentration of free aminoacids-taurine, GABA, glycine, phenylalanine, lysine, serine and aspartic acid.

(vi) Quantitative estimation of the rate of lipid-peroxidation after the administration of organophosphate-pesticide dichlorvos in different regions of the rat brain, cerebral hemisphere, cerebellum and brain stem.

(vii) Histochemical evaluation of acetylcholinesterase, succinic dehydrogenase and cytochrome oxidase activity.

(viii) Ultrastructural study of the different regions of the rat brain in organophosphate-dichlorvos-toxicosis, in particular, hypothalamus, hippocampus, cerebellum and spinal cord.

MATERIAL AND METHODS

2. MATERIALS AND METHODS

2.1. Organophosphorus compound and other chemicals:

The organophosphorus compound used in this study was O,O-dimethyl 2,2-dichlorovinyl phosphate-dichlorvos, as already described in introduction No. 1.17. Other chemicals especially the standard sample of amino acid (gamma aminobutyric acid (GABA), Taurine, Serine, Glycine, Lysine, Phenylalanine and Aspartic Acid) were purchased from B.D.H. (England). Monoamines standard were purchased dopamine (E. Merck, Germany); norepinephrine and 5-hydroxytryptamine (Sigma). Acetylcholine was purchased from V.P. Chest Institute, New Delhi. Other chemicals were purchased from B.D.H., Sarabhai and E. Merck Chemicals (India) of analar grade. All glass double distilled water was used in all the experiments.

2.2. Preparation of Organophosphorus compounds:

2.2.1. Preparation of diisopropyl fluorophosphate:

Diisopropyl fluorophosphate can be prepared by the action of $POCl_3$ on isopropanol, chlorinating the resulting intermediate and converting the diisopropyl chlorophosphate by means of sodium fluoride.

Reagents Used:

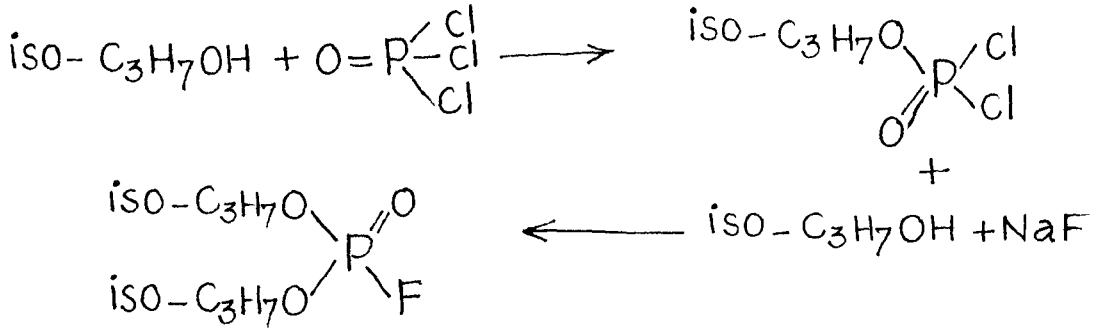
A. Isopropanol (dry)

B. Phosphorus oxychloride ($POCl_3$)

C. Carbon tetrachloride

D. Sodium fluoride.

METHOD: 38.0 ml (0.5 mol) dry isopropanol was added slowly at 30-35°C to 46 ml (0.5 mol) $POCl_3$ in CCl_4. Reaction occurred with evolution of HCl and was completed after standing overnight. Removal of the solvent under reduced pressure below 50°C gave isopropyl dichlorophosphate which was added to a mixture of 42 ml (0.55 mol) isopropanol and 75 gm (1.75 mol) sodium fluoride in dry benzene or carbon tetrachloride, with stirring at such a rate that the temperature was maintained at 45-50°C. Thereafter, it was refluxed for half an hour while stirring. Diisopropyl fluorophosphate (XXXVI) 55 gm was thus obtained. The sequence of reactions is shown in Scheme - 13.



SCHEME-13

2.2.2. Preparation of Coumarophosphate:

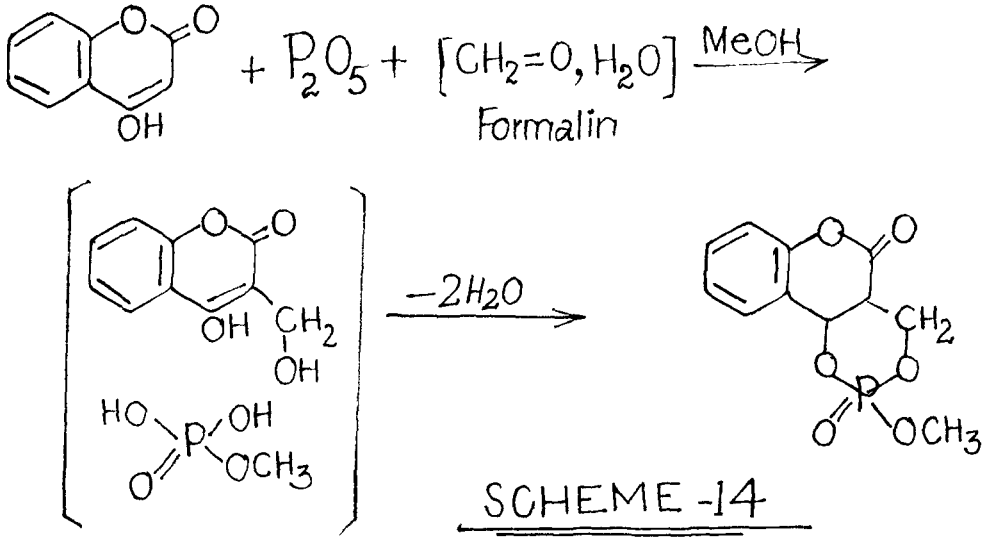
Coumarophosphates are the compounds having the phosphate nucleus and coumarin as a side linkage. They can be synthesized by the action of phosphorus pentaoxide with formaldehyde and coumarin in the presence of a solvent. The synthesis is accomplished as follow:

Chemicals Used:

1. Phosphorus penta oxide P_2O_5
2. Formaldehyde solution
3. 4-hydroxycoumarin
4. Methyl alcohol
5. Chloroform
6. Solvent ether
7. Absolute alcohol

METHOD: 1.42 gm (1 mole) P_2O_5 was taken in a three necked round bottom flask placed on ice. To this was added 1.62 gm (1 mole) 4-hydroxycoumarin dissolved in 10 ml methanol drop by drop so as to maintain the reaction at room temperature. Thereafter 1 ml (1 mole) formaldehyde solution was added dropwise, taking care that the temperature did not rise. This mixture was dissolved in methanol and was stored overnight by fitting a drying tube to the flask. Next morning, a white precipitate was obtained which was found by TLC to consist of the reaction product (XXXVII) contaminated with some 4-hydroxycoumarin, the starting material.

Separation was affected by taking up in the crude product the methanol in which the phosphate esters dissolved at room temperature while 4-hydroxycoumarin was sparingly soluble. Further purification was affected by crystallisation which gave the phosphate m.p. 182-184° as shown in Scheme - 14.



SCHEME -14

2.3. Experimental Animals:

Two hundred and fifty male albino rats of Charles-Foster strain weighing 150 ± 30 (S.D.) g, obtained from central animal house, J.N. Medical College, A.M.U., Aligarh, Central Drug Research Institute and I.T.R.C. colony (Lucknow), were used in this study. They were permitted free access to pellet diet (Hind Lever Laboratory Feeds, India) and tap water upto the time of experiment. The pellet composition corresponded with the nutritional standards recommended by the U.S. National Research Council's Publication No. 990, entitled "Nutritional Requirements of Laboratory Animals". The 'experimental' and 'control' groups consisted of equal number

of animals. Each group usually comprised of 10 rats, chosen at random, but no occasion less than 6 animals were used for a given experiment. Illumination in the colony room was maintained on a schedule of 12 h light, 12 h dark.

2.4. Administration of Organophosphorus Compound:

Dichlorvos (DDVP; 0,0-dimethyl, 2;2-dichlorovinyl phosphate - Nuvan 100 EC, Purity, 96.3%) was obtained from Ciba Geigy India Ltd. Organophosphate dissolved in normal saline and the rats of the experimental group were injected with aqueous solution of organophosphate intraperitoneally in a dose of 0.6, 1.5 and 3.0 mg/kg body weight daily for 3-15 days. The control group of animals were treated in an identical manner with equal volume of physiological saline.

2.5. Behavioural study:

2.5.1. Motor Activity:

The motor activity was recorded on two Animex Activity Meters (LKB-FARAD type DF-Sweden). Each activity meter which was preadjusted to equal sensitivity, consisted of six circular electromagnetic fields. Each rat was put in the plexiglass cage on top of the meter. When it crossed the electromagnetic field, a count was recorded on the Animex Recorder which was connected to the meter. The total count of each minute was printed. The activity meter was placed in a ventilated sound proof, lighted chamber, size

10' x 5' x 4'. The temperature of the chamber was kept constant throughout the experiment. The recorders were placed in a separate sound proof chamber to avoid disturbance to the rats.

Two groups of ten rats each were taken for recording the motor activity. One group served as control and was injected daily with 0.2 ml/100 gm body weight physiological saline i.p. The other group was administered the same volume of dichlorvos (3 mg/kg body weight) daily for 10 days. The motor activity was recorded each day for four hours in both the groups. The two channels of the Activity Meters were adjusted on different sensitivities so that one channel recorded only the gross movements and the other the total movements. The difference of the two showed the record of the fine movements.

2.5.2. Open Field Test:

The apparatus used for the 'Open Field Test' was similar to that used by Holland and Gupta (1966). Briefly it consisted of a circular open arena made up of wood measuring 32 ½ inches in diameter, enclosed by a wall 12 ½ inches high. The floor, which had a wooden surface, was marked out in three concentric circles divided into segments by lines radiating from the centre. The marking provided 25 floor units of approximately equal size and were used to score the ambulation of the animals moving around in the arena during the test. In the 'Open Field Test' two types of stimuli were presented to the animals: White noise with an intensity

of 78 dB (Ref. Intensity 0.0002 dynes/cm^2) was presented by a oscillator through 4 loudspeakers and light of the intensity of 165 foot - candles was produced by four photographic lamps. A translucent glass screen enclosed the arena on all the sides, the front side having a glass door through which the subject was placed in the arena and the investigator observed the rat under test. Ambulation, rearing and grooming responses were recorded by a three channel hand operated counter. The ambulation response was defined as a 'walking around' score derived from the number of radial segments of the arena which were crossed by the subject. The placement of all the four limbs in one segment was taken as one unit of ambulation. One rearing score was awarded when the rat stood on hind limbs with the support of the side wall and two for standing without the support. Grooming response was determined by the number of times the animal scratched its face by fore limbs. Defecation score was the number of faecal boluses deposited by the animal on the floor during the test.

The experimental design for 'Open Field test' comprised of two groups of ten rats each. Dichlorvos was administered in a dose of 3 mg/kg body weight intraperitoneally (i.p.) daily for 10 consecutive days to group 1. Concurrently, the same volume of physiological saline was administered i.p. to rats of group 2. An absorption period of 15 minutes was allowed in the home cage before

the animal was exposed to the test. Each animal was tested daily for 2 minutes and the ambulation, rearing, preening and defecation score was recorded.

2.6. Removal of brain and spinal cord:

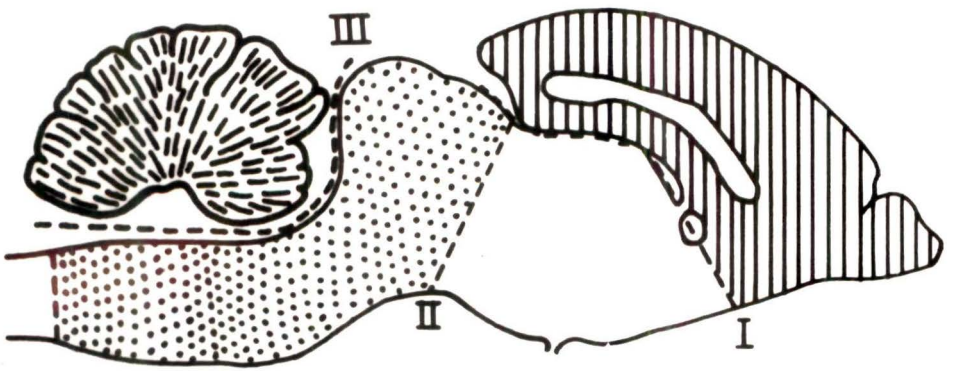
The rats were sacrificed by decapitation. The brains and cervical spinal cord were removed rapidly and blotted on filter paper. The brains were dissected out in a cold room at a temperature of 10 ± 2°C into; cerebral hemisphere, cerebellum and brain stem as shown in Fig. 4 and weighed to the nearest milligram on a electrical balance and separately homogenized for the biochemical analysis.

2.7. Biochemical estimation of acetylcholinesterase:

The activity of acetylcholinesterase was assayed at 37°C according to the method of Hestrin (1949). It was expressed as µ moles acetylcholine hydrolysed/gm fresh tissue/hr. The following reagents were required for this method:

Explanation of Figure:

Fig. 4 : Diagrammatic representation of plane of direction of cerebral hemisphere, cerebellum and brain stem.



:CEREBRAL HEMISPHERE

:CEREBELLUM

:BRAIN STEM

1) Hydroxylamine: 2 M - Hydroxylamine hydrochloride prepared in distilled water and should be stored in cold.

2) Alkaline solution: 3.5 N Sodium hydroxide (NaOH).

3) Acid solution: Concentrated HCl (Sp. gr. 1.18) diluted with 2 parts by volume of water.

4) Ferric chloride: 0.37 Ferric chloride ($FeCl_3$: 6 H_2O) prepared in 0.1 - N-HCl.

5) Buffer substrate: Acetylcholine hydrochloride, 0.04 M (0.7625 gm/100 ml distilled water) is diluted 10 times in 0.06 M Phosphate buffer (pH 7.2) and stored in refrigerator.

6) Phosphate buffer: (0.1M; pH 8.0) - 94.7 ml of 0.1M Na_2HPO_4 was added to 5.3 ml of NaH_2PO_4 and diluted to a total of 200 ml with distilled water.

7) Alkaline-hydroxylamine reagent: Alkaline hydroxylamine reagent was prepared freshly before use by mixing equal volumes of reagent 1 and 2. The mixture was kept for about 3 hours at room temperature before used.

Procedure:

(i) Standardization of acetylcholine: The standard solution of different concentration of acetylcholine in phosphate buffer was prepared, then 0.1 - 1.0 ml of standard solution of acetylcholine according to the concentration were taken and make it upto 1.0 ml with phosphate buffer. Add 2.0 ml of alkaline-hydroxylamine reagent. After one minute the pH of the reaction mixture was brought to 1.2 ± 0.2 with 1.0 ml of acid solution. Then 6 ml of Ferric chloride solution was added to each tube. A purple brown colour developed which was read at 540 nm. The graph was plotted on different concentration of acetylcholine against the optical density.

(ii) Assay of acetylcholinesterase: Animals were divided into four groups. Group 1, 2 and 3 consisting of 10 rats each, were administered dichlorvos in a dose of 0.6, 1.5 and 3.0 mg/kg body weight i.p. respectively daily for 10 days. The fourth group comprised of 15 rats served as a control and was concurrently injected with equal volumes of physiological saline. The animals were decapitated and their brain and cervical spinal cord was dissected out into cerebral hemisphere, cerebellum and brain stem in a cold room, weighed to the nearest milligram on a electrical balance and roughly homogenized in chilled phosphate buffer (0.1M; pH 8.0). In 0.1 ml of homogenate add 0.5 ml phosphate buffer substrate. Incubated the reaction mixture at 37°C for 15 minute. After incubation added 2.0 ml of alkaline

hydroxylamine reagent, then the pH of the reaction mixture was brought to 1.2 ± 0.2 with 1.0 ml of acid solution. In the added 6 ml of colouring reagent (ferric chloride solution). A purple-brown colour was developed which was read at 540 nm. If there was any turbidity in the mixture, centrifuge it and then read. The rate of acetylcholine hydrolysed was calculated from the standard curve.

2.8. Determination of Amino acids concentration:

All the amino acids were estimated according to the paper chromatography method of Awapara's (1948) and the values of each amino acids was calculated by the standard curve of each amino acid individually.

Standardization of Amino acids: Standard solutions of different concentration of amino acid were prepared and applied to the Whatman filter sheet No.1 size 18" x 12" for chromatography and run for 18 hours in a chromatography chamber in the solvent (Butanol: Glacial acetic acid and DD water in the ratio of 4:1:1) system. After 18 hours the filter sheet was taken out and dried in air. It was sprayed with ninhydrin solution, spot of each individual amino acid was developed within half an hour. The spotted part was taken out of the filter sheet and dissolved in 5 ml (DD water and absolute alcohol 1:1) by heating for 10-15 minute in a boiling water bath. The optical density was read at 660-750 nm in Carlzeiss Spekol (W. Germany) spectrophotometer. The graph of each amino acid

was plotted. Values of each amino acid in control and experimental group were calculated by the standard curve.

Assay of Amino acids: The following reagents are required for the estimation of amino acids:

1. 0.01 N Hydrochloric acid (Analar grade)
2. Absolute alcohol (Pure) (Analar grade)
3. 75% Ethyl alcohol (Analar grade)
4. Methyl alcohol (Analar grade)
5. Chloroform (Analar grade)
6. Ninhydrin: 0.5% solution of ninhydrin in 95% ethyl alcohol.
7. D.W. Water, absolute alcohol mixture of ratio 1:1.

Animals were divided into two groups of 15 rats each. Rats of group 1 injected organophosphate-dichlorvos 3 mg/kg body weight intraperitoneally daily for 15 days. The group 2nd served as a control and recieved equal volume of physiological saline i.p. concurrently. The animals were decapitated after 15 days and their brains and cervical spinal cord were removed. The brains were dissected into; cerebral hemisphere, cerebellum and brain stem in a cold room, weighed to the nearest milligram on an electrical balance and were homogenized in 80% chilled ethyl alcohol. The homogenates were treated according to the method of Awapara (1948) for the estimation of free amino acids. Following storage for an hour on ice, the homogenates were centrifuged at 16,000 g at 0°C on the International Refrigerated ultracentrifuge for 10 minutes. The precipitate was washed with

3.5 ml ethyl alcohol and the supernatant was evaporated to dryness on the water bath at 70-90°C. To the residue was added 1 ml distilled water, 2 ml methyl alcohol and 2 ml chloroform and the resultant solution centrifuged on clinical centrifuge for 20 minutes. The supernatant was obtained which was applied to chromatographic paper as recommended by Awapara (1948) and the known standard amino acid solution were placed on the same sheet for further separation by paper chromatography in the same solvent as used in case of standardization of amino acids. The value of amino acid was calculated in u mole/g fresh brain weight.

2.9. Estimation of monoamine levels:

Estimation of dopamine, norepinephrine and 5-hydroxytryptamine was done according to the method of Welch and Welch (1969).

Reagents and glassware:

1) n-Heptane was washed with one fifth volume of 1N NaOH, then with 1 N-HCl as described by Shore and Olin (1958).

2) n-Butanol (fluorometric grade) was used without further purification.

3) Diethyl ether was washed with a saturated solution of $FeSO_4$ to remove accumulated peroxides and subsequently with distilled water to remove the $FeSO_4$.

4) Phosphate buffer (0.5 M; pH 7.3) - 77 ml of 0.5 M Na_2HPO_4 was added to 23 ml of 0.5 M KH_2PO_4.

5) Ethylenediamine was distilled and stored in a dark bottle in the cold.

6) Acetate buffer (2 M; pH 6.0) - 2 M acetic acid was adjusted with 2 N - NaOH to a pH 6.0.

7) Iodine solution; 0.1 N Iodine (3.175 gm) was dissolved in distilled water containing 12.5 gm KaI, diluted to 250 ml and stored in a dark bottle in the cold.

8) Sodium thiosulfate; 0.1 N, $Na_2S_2O_3.5H_2O$ (6.2 gm) was dissolved in distilled water, made to 250 ml, and stored in a dark bottle in the cold.

9) EDTA, 10% (W/V) Ethylenediamine tetra-acetate disodium salt (10 gm) was dissolved in distilled water with heating and dilute to 100 ml.

10) Alkaline sulfite/EDTA solution: $Na_2SO_3.7H_2O$ (12.6 gm) was dissolved in 25 ml of 10% EDTA and diluted to 250 ml with 5-N NaOH.

11) Alkaline ascorbic acid/ethylenediamine solution: Immediately before use, ascorbic acid (200 mg) was dissolved in 2.5 ml 0.01 N - HCl, and added to 22.5 ml 10 N - NaOH containing 0.5 ml redistilled ethylenediamine. Since the solution was very viscous, it was necessary to mix it thoroughly.

12) HCl/acetic acid reagent: Concentrated HCl and glacial acetic acid were mixed in a 1:1 ratio.

13) Ascorbic acid solution: Immediately before use, 100 mg ascorbic acid was dissolved in 10 ml 0.01 N - HCl.

All chemicals were reagent grade. Reagents and distilled water were stored in hard glass bottles with glass stoppers, since fluorescent contaminants are leached from plastic and rubber stoppers. Glassware was kept separate from that used in other procedure, after thorough washing it was rinsed exhaustively with tap water and then with glass-distilled water. Glass-stoppered bottles sized 30,60 and 125 ml, glass pipette and other glassware of corning grade were used for extraction and estimation of the monoamines.

Standardisation and recovery experiment of monoamines: Stock solution of dopamine, norepinephrine and 5-hydroxytryptamine were prepared in 0.01N-HCl at a concentration of 100 µg/ml of the free base. They were stored in the cold for upto one month. On the day of use, the stock solutions of DA, NE and 5-HT were diluted with 0.01 N-HCl to working standards having a concentration of 1 µg/ml. Then 10 ml each of the DA and 5-HT working standards and 5 ml of NE working standards were diluted together to 50 ml with 0.01 N HCl to obtained a "standard amine solution" containing 100 µg NE, 200 ug DA and 200 ug 5-HT/ml.

Ten control rats brains and spinal cord were dissected out. Brains were dissected into three parts: cerebrum, cerebellum and brain stem, and each part pooled individually and homogenized in 8 ml of 0.01N-HCl. One ml of this homogenate was added to each of 8 bottles (separately for each part) containing 25 ml n-butanol and 4 gm NaCl. Two of these tissue samples were carried through the extraction procedure without

added standard; the others were prepared as internal standard by adding 0.5; 1.0 and 1.5 ml of the "standard amine solution" to duplicate bottles. The internal standard covered the range of 50, 100, 150 µg for 5T and the range of 100, 200 and 300 ug for DA and 5-HT. Each bottle received 0.1 ml 10% EDTA and enough 0.01 N-HCl to make a total volume of 3.1 ml, and the internal standard were then extracted exactly as the other brain samples. Duplicate treatment blanks containing only HCl and EDTA were also carried throughout the entire procedure. Recovery was estimated by comparison with unextracted standards that were freshly prepared and analyzed simultaneously with the extracted samples. For standardization of monoamine, prepared the solution of different concentration (200 - 300 µg)/ml. Solution were taken in different test tube ranging in volume from 0.1 - 1.0, make it upto 1.5 ml and carried the entire procedure for fluorometric analysis. The fluorescence was read at different excitation/emision. The standard curves of each monoamine was plotted. Values of each monoamine in control and experimental group were calculated by these standard curves.

Estimation of monoamines: Animals were divided into five groups of 10 rats each. Group 1,2,3 and 4th were administered organophosphate-dichlorvos in a dose of 3 mg/kg body weight i.p. daily for 3,5,7 and 10 days respectively. The group 5th served as a control and was concurrently injected with equal volumes of physiological saline. The animals were decapitated after 3,5,7 and 10 days and their cerebral hemisphere, cerebellum, brain stem and cervical

spinal cord were rapidly dissected out in a cold room at a temperature of 10 ± 2°C. They were weighed to the nearest milligram and were separately homogenized.

Extraction: Parts of the brain and spinal cord were homogenized in 1.5 ml ice cold 0.01N HCl to which 0.1 ml 10% EDTA had been added. The homogenate, followed by a 1.5 ml rinse of 0.01N HCl, was added to 25 ml n-butanol in a 60 ml glass-stoppered bottle containing 4 gm NaCl. The bottles were shaken for 10 minutes on a reciprocating shaker at about 250 excursions per minute and centrifuged at 3000 r.p.m. for 8 minute in the cold. The butanol (24 ml) was decanted into a 125 ml glass-stoppered bottle containing 40 ml n-heptane, 1.5 ml of 0.5M pH 7.3 phosphate buffer was added to the heptane/butanol mixture; the bottles were shaken again for 10 minute and centrifuged at 2000 r.p.m. for 8 minute in the cold. At this stage, it was important that the pH of the phosphate buffer not be allowed to drop below 7.0, otherwise the recovery of monoamine was greatly reduced. Phosphate buffer (1.5 ml), which increased slightly in volume due to water driven from the butanol phase after the addition of heptane, was transferred to a clean 30 ml bottle and was acidified with 3-N-HCl to pH 3.5 - 4.0. Then 20 ml of peroxide free ether was added and the bottles were shaken for 10 minute and centrifuged in the cold. Three 0.5 ml aliquots of the acid-aqueous layer were taken directly from the bottom of the ether extraction bottles with a 0.5 ml volumetric pipet and were refrigerated. 5-HT was estimated on the same day, whereas NE and DA were estimated on the following day. The could be safely stored over night under refrigeration.

Fluorometric Analysis: The fluorometric analysis of NE, DA and 5-HT was carried out on Zeiss Spectrofluorometer set up C (Carl Zeiss, West Germany). The fluorescence of monoamines was recorded on the following excitation and emission wave lengths:

	Excitation	Emission
NE	400	510
DA	335	380
5-HT	295	535

Norepinephrine: By adding reagents at 4-5 second intervals, upto 60-75 sample could be analyzed together against the same standards. One should wait for approximately 5 minute interval after the addition of each reagent to allow completion of the reaction, although slightly longer or shorter period did not seem to make a significant difference. To the 0.5 ml samples collected in small test tuber, the following were added in order: 0.5 ml 2 M pH 6.8 acetate buffer, 0.1 ml Iodine solution, 0.15 ml 0.1 N $Na_2S_2O_3.5H_2O$ and 0.2 ml alkaline ascorbic/ethylenediamine solution. The last reagent was very viscous so it must be thoroughly mixed. The final sample volume was 1.45 ml. The test tube rack was placed under a fluorescent desk lamp with the top of the tube positioned about 5-10 cm from the reflector, maximal fluorescence was developed within 35 minute. Fluorescence was read at given excitation/ emission using 2 mm slits.

Dopamine: To the 0.5 ml sample, the following reagents were added, at 5 minute intervals: 0.5 ml acetate buffer 2M, pH 6.8; 0.1 ml 0.1N Iodine solution; 0.2 ml alkaline sodium sulfite/EDTA solution; and 0.25 ml 1:1 glacial acetic acid/concentrated HCl reagent. The final sample volume was 1.5 ml and pH was 3.8-4.2. All the test tubes were placed in a boiling water bath for 45 minute. After which they were allowed to cool at room temperature before reading. Fluorescence was read at given excitation/emmission using 2 mm slits within one hour after development.

5-Hydroxytryptamine: To each of the 0.5 ml samples, obtained in the extraction procedure, was added 1.5 ml of 6 N-HCl. One sample was acidified at a time and its fluorescence was read promptly at given excitation/emmission using 2 mm slits.

2.10. Rate of lipid-peroxidation assay:

Rate of lipid-peroxidation were estimated according to the method of Utley et al. (1967). The following reagents were required for this method:

(1) 0.15M - KCl: 2.2368 gm potassium chloride dissolved in 200 ml double distilled water.

(2) 10% (W/V) TCA: 10 gm Trichloroacetic acid (TCA) was dissolved in 100 ml double distilled water.

(3) <u>0.67% TBA (W/V)</u>: Thiobarbituric acid (TBA) was prepared by adding two pellet of sodium hydroxide to make the water alkaline. 2-Thiobarbituric acid (TBA) was weighed (0.67 gm) and added in alkaline water. TBA was dissolved by slow stirring and make it upto 100 ml with double distilled water.

Animals were divided into four groups of 10 rats each. Group 1,2 and 3 were administered organophosphate-dichlorvos 3.0 mg/kg, 1.5 mg/kg and 0.6 mg/kg body weight respectively i.p. daily for 10 days. The group 4th served as a control was sham injected with equal volumes of physiological saline i.p. concurrently. The animals were fasted overnight (with water ad libitum), sacrificed by decapitation and their brains were taken out. Brain was dissected out into; cerebral hemisphere, cerebellum and brain stem. All parts of the brain were homogenized (10%, W/V) in chilled 0.15M potassium chloride. For assay of lipid peroxidation, 1.0 ml of homogenates were incubated at $37^{\circ} \pm 1^{\circ}C$ in a metabolic shaker (120 strokes/min.; amplitude 1 cm) for 3 hours. After incubation 1 ml of 10% TCA was added for the precipitation of protein. The reaction mixture was centrifuged at 800 g for 10 minutes. One millilitre of the clear supernatant was mixed with 1 ml 0.67% thiobarbituric acid and 1 ml double distilled water. Placed the test tubes in a boiling water bath for 10 minutes, cooled and the absorbance of the colouring solution was read at 535 nm. The rate of lipid peroxidation expressed as n mole of malonal-dehyde formed/30 minutes using extinction coefficient 1.56×10^5 as described by Utley <u>et al.</u> (1967).

Lipid peroxidation was calculated using the following formula:

n moles of malonaldehyde formed/30 minute (X)

$$X = \frac{\triangle \text{ O.D.} \times 30 \times 1000 \times 1000 \times 1000 \times 3 \times 2}{1.56 \times 100000 \times 1000 \times 180}$$

$$X = \frac{\triangle \text{ O.D.} \times 10}{1.56}$$

Where $\triangle$ O.D. = change of optical density at zero hour and three hour incubation.

2.11. Light Microscopy:

Twenty rats were divided into two groups of 10 each. Rats of group 1 were administered dichlorvos 3 mg/kg body weight i.p. daily for 10 days and equal volumes of physiological saline was concurrently injected to the second group. The animals were sacrificed by decapitation and their brain was dissected out for the histochemical demonstration of the following enzymes:

(i) **Acetycholinesterase:** The activity of acetylcholinesterase was demonstrated histochemically according to the method of Koelle (1951) which was modified by Gomori (1952).

Stock solution was prepared as follow:

Copper sulphate ($CuSO_4.5\ H_2O$) - 0.3 gm

Glycine	-	0.375gm
Magnesium chloride ($MgCl_2.6\ H_2O$)	-	1.00 gm
Maleic acid	-	1.75 gm
4% NaOH solution	-	30.00 ml
Saturated (about 40%) Na_2SO_4 solution.	-	170.00 ml

Stored the solution in refrigerator for at least three months. In fridge the sodium sulphate may crystallized but that does not affects result.

Preparation of incubating solution:

Dissolved 20 mg acetyl thiocholine iodide in a few drops of distilled water and make it upto 10 ml with stock solution.

Procedure:

Freely floating frozen sections, 20-30 µ thick (cut with a Kryoton 1310 K, Leitz, W. Germany) were used for the histochemical demonstration of acetylcholinesterase.

- Sections were incubated for 40 minutes at 37°C in an incubating medium.
- Rinsed in 2 or 3 change of saturated aqueous sodium sulphate solution.
- Placed the sections in dilute solution of yellow ammonium sulphide for 2 minutes.

- Rinsed in distilled water for 2 minutes.
- Mount in glycerine-jelly (Sigma Chemical Co.).

(ii) Cytochrome Oxidase: The histochemical demonstration of cytochrome oxidase activity was based on the technique mentioned in Sigma Technical Bulletin No. 185 revised in December, 1973. Kit No. 185-A containing the following reagents was obtained from Sigma Chemical Co., St. Louis (U.S.A.):

- 8-Amino-1,2,3,4-Tetrahydroquinoline, Grade III (Stock No. 185-2) - 50 mg ampoule was dissolved in 0.5 ml ethanol and the resultant solution transferred to a 125 ml Erlenmeyer flask.
- Contents of one capsule (15 mg) of p-Aminodiphenyl - amine were added and swirled to dissolved.
- 35 ml distilled water was added (solution became cloudy) and 15 ml Trizma buffer (Stock No.106-74) was added and shaken well. The solution was centrifuged and supernatant fluid transferred to a coplin jar.
- Mounted frozen sections of different parts of the rat brain were immersed in this coplin jar and incubated at room temperature for 15 minutes.
- Mounted sections were then transferred to another coplin jar containing cobalt-formalin solution (Reagent-H). Chelation-fixation was allowed for 1 hour.

- Slides were washed in tap water for 5-10 minutes.

- Sections were mounted in Glycerol-Gelatin (Stock No. CG-1, Sigma Chemical Co., St. Louis).

(iii) Succinic dehydrogenase: The activity was determined according to the method described by Burpiva (1963). Freely floating frozen sections, 15-20 µ thick (Cut with a Kryotom 1310 K, Leitz, W. Germany) were incubated for 30 minutes at 37°C in an incubating medium prepared as followed:

- Mixed 2.5 ml of phosphate buffer (0.06M; pH 7.4) with 0.3 ml cobalt chloride (0.5M) and filtered off the precipitate.

- Added 2.5 ml sodium succinate (0.2M) solution, 0.05 ml $CaCl_2$ solution (0.33M), 0.5 ml $NaHCO_3$ (0.06M), 0.4 ml $AlCl_3$ solution (0.01M) and 2.5 ml NBT solution (1 mg NBT salt per ml.).

- Adjusted the pH to 6.4 and diluted to 10.45 ml with distilled water.

Sections were fixed in formol-calcium solution (Baker et al. 1946) for 10 minutes and counter stained with 0.1% methyl green. The sections were mounted in glycerol-gelatin (Sigma Chemical Co., St. Louis) containing 11.9 gm $CoCl_2.6H_2O$ per 100 ml.

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3. RESULTS

3.1. IR and NMR of organophosphorus compounds:

The i.r. of the coumarophosphate showed the carbonyl band of the coumarin moiety at 1720-1730 cm^{-1} and the nmr spectrum apart from signals of aromatic protons, a sharp singlet at 6.2 τ which could arise only from a methoxy group. The methylene protons are not clearly resolved but their quartet is discernible at 4.7 τ .

3.2. Physical signs:

During the period twenty out of 250 animals died of toxicity at varying time intervals. Failure to gain optimum weight was noted in all the poisoned animals. Whereas the mean weight gain in the control group of rats was 20 $\pm$ 4 g, in the poisoned animals it was only 2 $\pm$ 0.5 g. The signs of toxicity in the poisoned animals included irritability, salivation, muscular twitchings, hyper-excitability to tactile and at times fasciculations, dragging of hind limbs (6 animals), fits of abnormal rotation of head and neck and curving of the body to one side (in 10 cases) were observed.

3.3. Behavioural changes after organophosphate administration:

3.3.1. **Motor activity:** First two days of drug treatment did not produce a change in motor activity. Significant reduction was observed from third day onwards with a peak decrease in

activity on the seventh day (Table-8). This decrease was more pronounced in the gross movements ($P < 0.001$) as compared to the fine movements ($P < 0.01$). After the seventh day the activity showed a gradual recovery inspite of continued drug treatment. As shown in Table-8 and Fig. 5 the depression in motor activity was insignificant on the tenth day as compared to the placebo.

3.3.2. Open field behaviour: In the open field experiment, ambulation, rearing, preening and defecation scores were depressed after dichlorvos treatment (Figs. 6-9). Ambulation showed a mean control value of 22.0 ± 3.0. This was progressively reduced with a peak depression (2.60 ± 0.42) on the seventh day which was 12% of the control (Table-9). On the tenth day ambulation, though significantly depressed, showed a recovery to 30% of the control as compared to the depression on the seventh day. Rearing response was markedly reduced ($P < 0.001$) to 2% of the control on the seventh day and similar to ambulation showed a recovery to 18% on the tenth day. The mean preening scores were not reduced on the third and fifth day. Sixth day onwards there was a progressive decrease in the preening score which was highly significant ($P < 0.001$) on the tenth day. Defecation showed the patterns similar to ambulation and rearing with a maximum reduction on the seventh day (Fig. 9).

Explanation of Figure:

Fig. 5 : Total, fine and gross movements of albino rats when placed on activity meter for four hours (10.00 A.M. to 2.00 P.M.) daily for ten days after the administration of dichlorvos (3 mg/kg body weight i.p.). A, B and C are the control values of the total, fine and gross movements of the rats treated with physiological saline i.p. daily. Value of each day is the Mean ± S.E. burs of 10 animals.

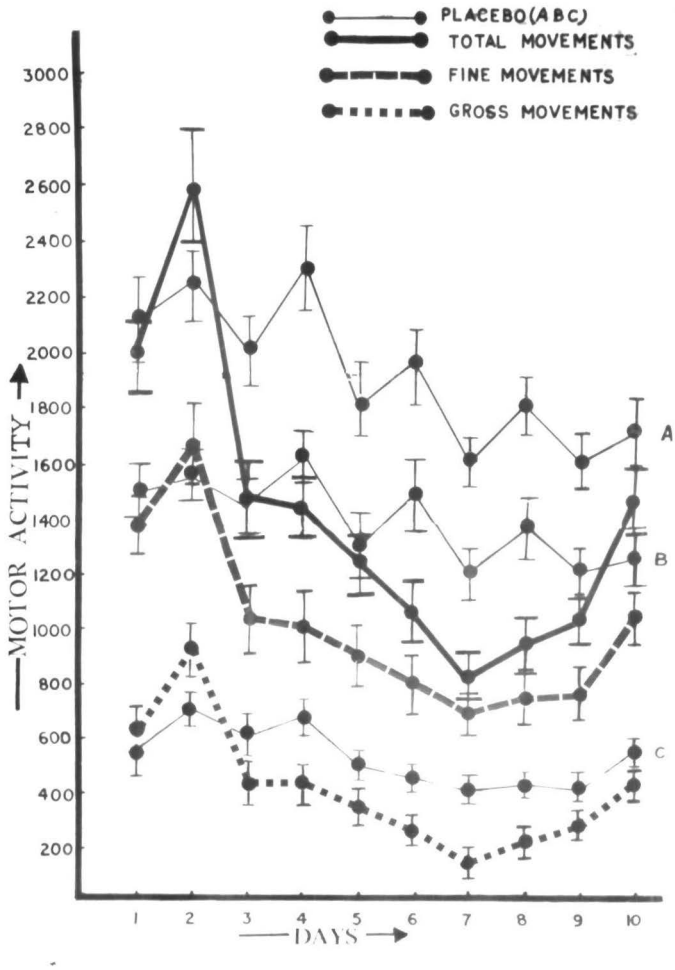

PLACEBO(A B C)
TOTAL MOVEMENTS
FINE MOVEMENTS
GROSS MOVEMENTS
MOTOR ACTIVITY
3000
2800
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
A
B
C
1
2
3
4
5
6
7
8
9
10
DAYS

Table - 8: Showing per cent alteration in the motor activity after dichlorvos administration (3 mg/kg body weight i.p.) as compared with the control value on the same day (control taken as 100%).

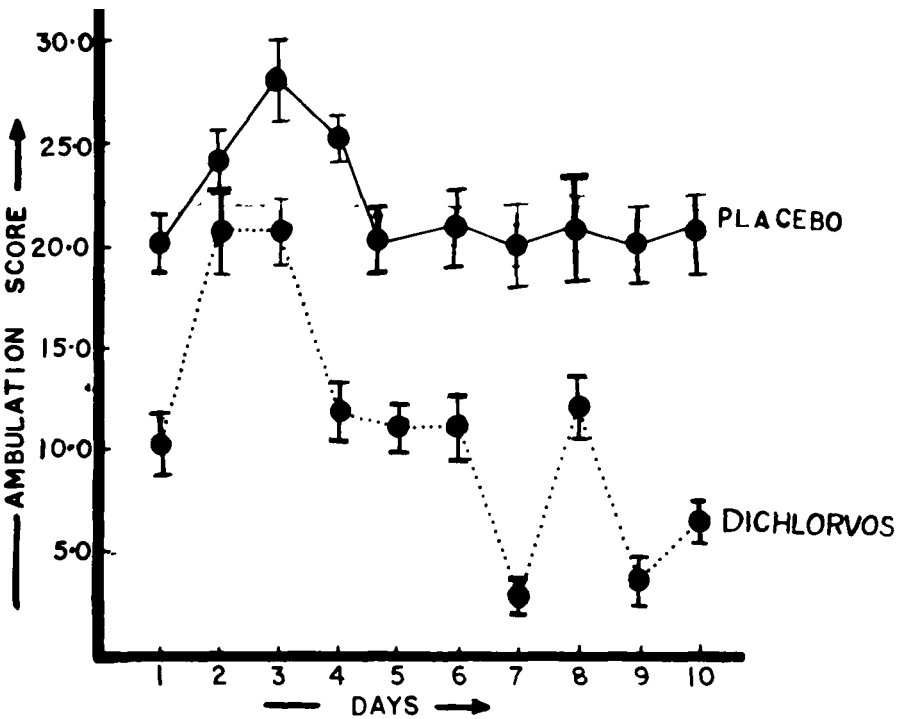
DAY	TOTAL MOVEMENTS	GROSS MOVEMENT	FINE MOVEMENTS
1	- 5	+ 14	- 12
2	+ 14	+ 24	+ 7
3	- 28*	- 30*	- 29*
4	- 38*	- 37*	- 40*
5	- 32*	- 33*	- 31*
6	- 47**	- 45**	- 47**
7	- 50***	- 69***	- 44**
8	- 49***	- 53**	- 47**
9	- 37*	- 34*	- 38*
10	- 16	- 7	- 19

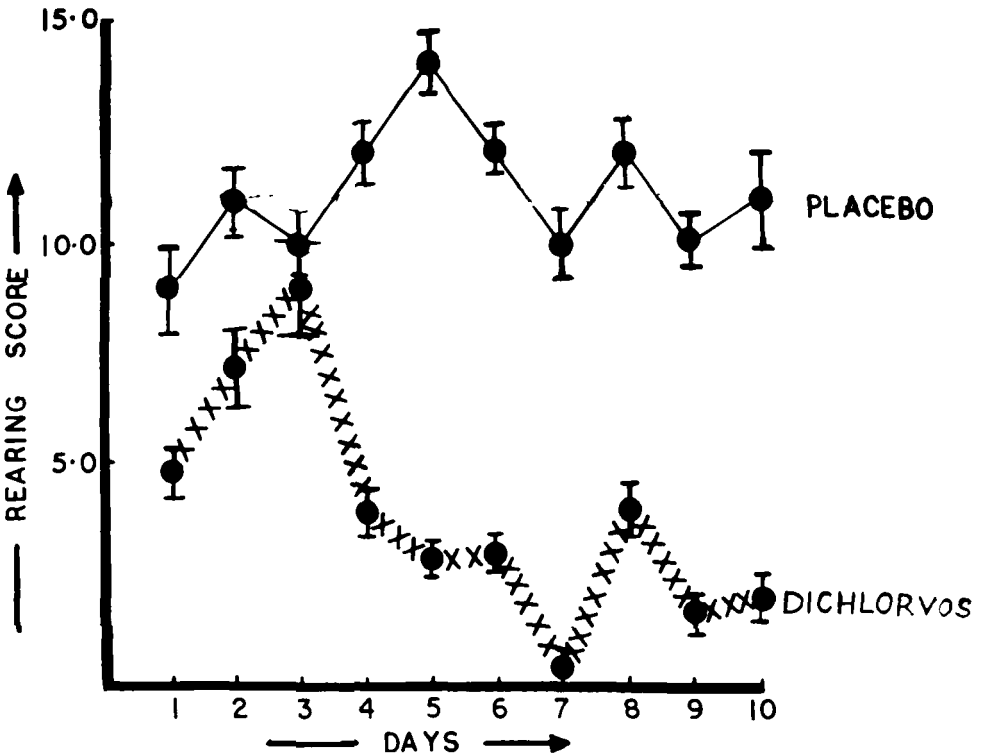
* Significantly different from control $P < 0.05$.
** Significantly different from control $P < 0.01$.
*** Significantly different from control $P < 0.001$.

Explanation of Figures:

Fig. 6 : Effects of dichlorvos (3 mg/kg body weight i.p.) on the ambulation score during the open field test of albino rats for two minutes daily for 10 days. Placebo group treated with physiological saline i.p. daily for the same period. Values of each day is the mean ± S.E. of 10 animals.

Fig. 7 : Effects of dichlorvos (3 mg/kg body weight i.p.) on the rearing score during the open field test of albino rats for two minutes daily for 10 days. Placebo group treated with physiological saline i.p. daily for the same period. Values of each day is the mean ± S.E. of 10 animals.


30·0
25·0
20·0
15·0
10·0
5·0
AMBULATION SCORE
PLACEBO
DICHLORVOS
1
2
3
4
5
6
7
8
9
10
DAYS


15·0
10·0
5·0
REARING SCORE
PLACEBO
DICHLORVOS
1
2
3
4
5
6
7
8
9
10
DAYS

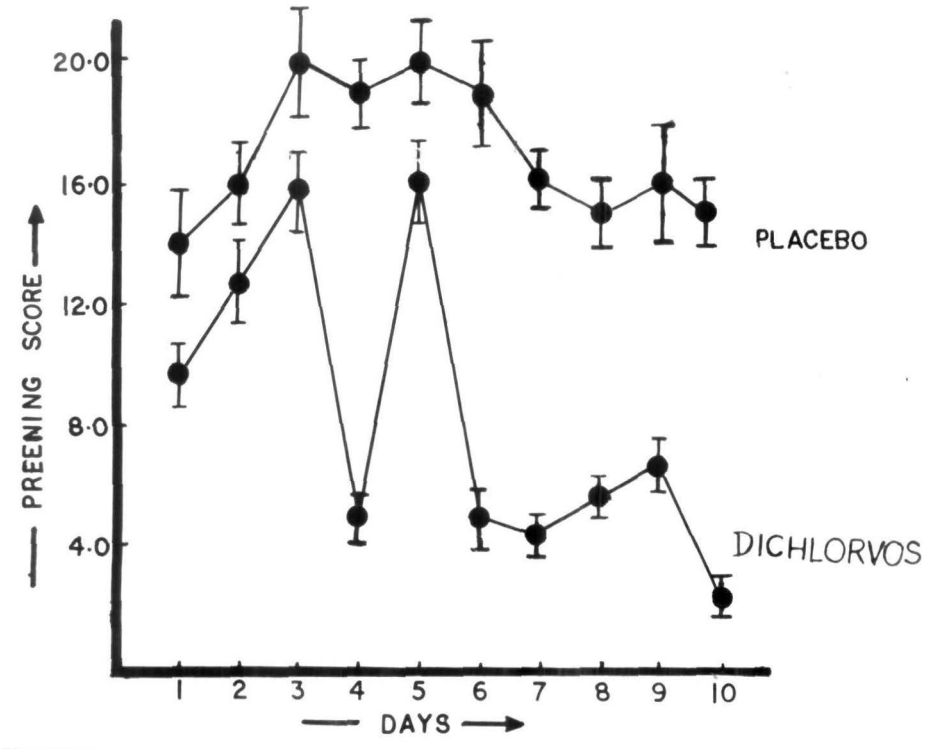
6

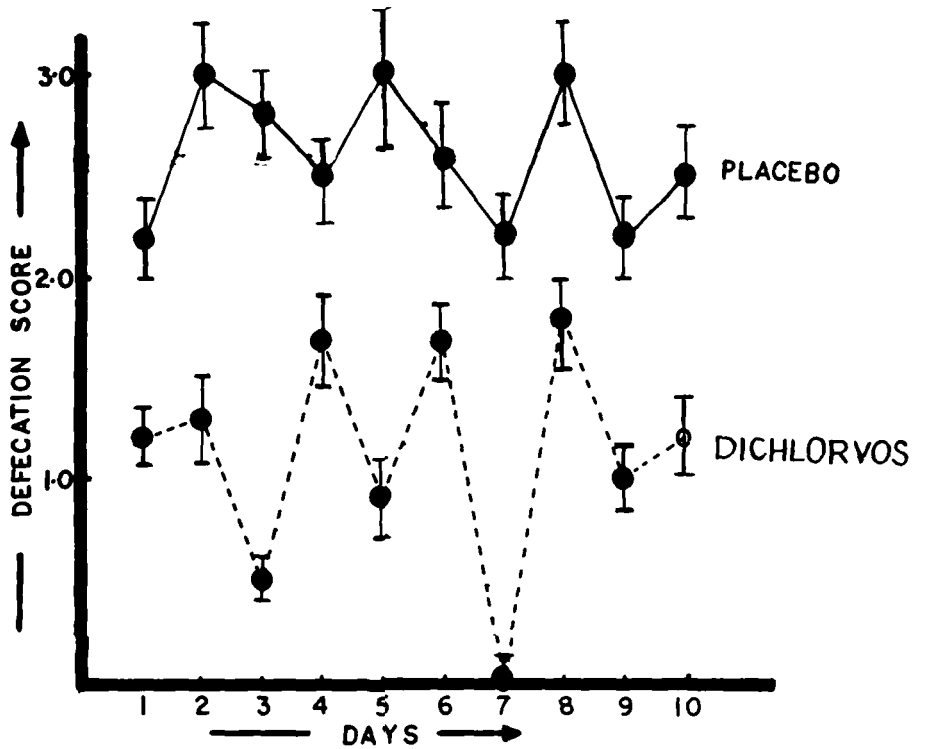
7

Explanation of Figures:

Fig. 8 : Effects of dichlorvos (3 mg/kg body weight i.p.) on the preening score during the open field test of albino rats for two minute daily for 10 days. Placebo group treated with physiological saline i.p. daily for the same period. Values of each day is the Mean ± S.E. bars of 10 animals.

Fig. 9 : Effects of dichlorvos (3 mg/kg body weight i.p.) on the defecation score during the open field test of albino rats for two minute daily for 10 days. Placebo group treated with physiological saline i.p. daily for the same period. Values of each day is the Mean ± S.E. bars of 10 animals.


20·0
16·0
12·0
8·0
4·0
PREENING SCORE
PLACEBO
DICHLORVOS
1
2
3
4
5
6
7
8
9
10
DAYS


3·0
2·0
1·0
DEFECATION SCORE
PLACEBO
DICHLORVOS
1 2 3 4 5 6 7 8 9 10
DAYS

8

9

Table - 9: Showing ambulation, rearing and preening scores after dichlorvos treatment (3 mg/kg body weight i.p.) and the effect as per cent of mean on 3rd, 5th, 7th and 10th day.

	AMBULATION		REARING		PREENING	
	Mean	% of Mean	Mean	% of Mean	Mean	% of Mean
Control	22.00 ± 3.00	100	11.00 ± 2.80	100	15.40 ± 2.86	100
Third day	20.80 ± 2.78	95	9.00 ± 1.26	81	15.90 ± 2.88	103
Fifth day	11.00 ± 2.47*	50	2.80 ± 0.87*	25	16.10 ± 2.91	104
Seventh day	2.60 ± 0.42***	12	0.25 ± 0.06***	2	4.25 ± 0.96**	28
Tenth day	6.50 ± 1.60**	30	2.00 ± 0.21**	18	2.25 ± 0.41***	14

* Significantly different from control $P < 0.05$.
** Significantly different from control $P < 0.01$.
*** Significantly different from control $P < 0.001$.

3.4. Activity of acetylcholinesterase in organophosphate intoxication:

The activity of acetylcholinesterase is expressed as µ moles of acetylcholine hydrolysed/g fresh tissue/hr. The rate of hydrolysis of acetylcholine was calculated from the standard curve of acetylcholine (Fig. 10). The activity of acetylcholinesterase in different regions of the rat brain and spinal cord after the administration of various doses of organophosphate-dichlorvos is presented in Figs.11-13. A dose-dependent inhibition of acetylcholinesterase was observed (Fig. 14). At the dose of 0.6 mg/kg the cholinesterase activity was significantly inhibited ($P < 0.05$) in all regions of the brain and spinal cord, whereas at 1.5 mg/kg the inhibition in cerebral hemisphere and brain stem was more ($P < 0.01$). However, when the dose was increased to 3.0 mg/kg the level of significance was correspondingly higher ($P < 0.001$).

3.5. Amino acids investigation:

Concentration of amino acids was calculated from the individual standard curves. Figs. 15-21 show the standard curve between the optical density and the concentration of all the seven amino acids: Gamma amino butyric acids, lysine, taurine, aspartic acid, serine, glycine and phenyl alanine.

The values of amino acids have been expressed in µ mole/g of fresh tissue. Figs. 22-25 show the effects of organophosphate-

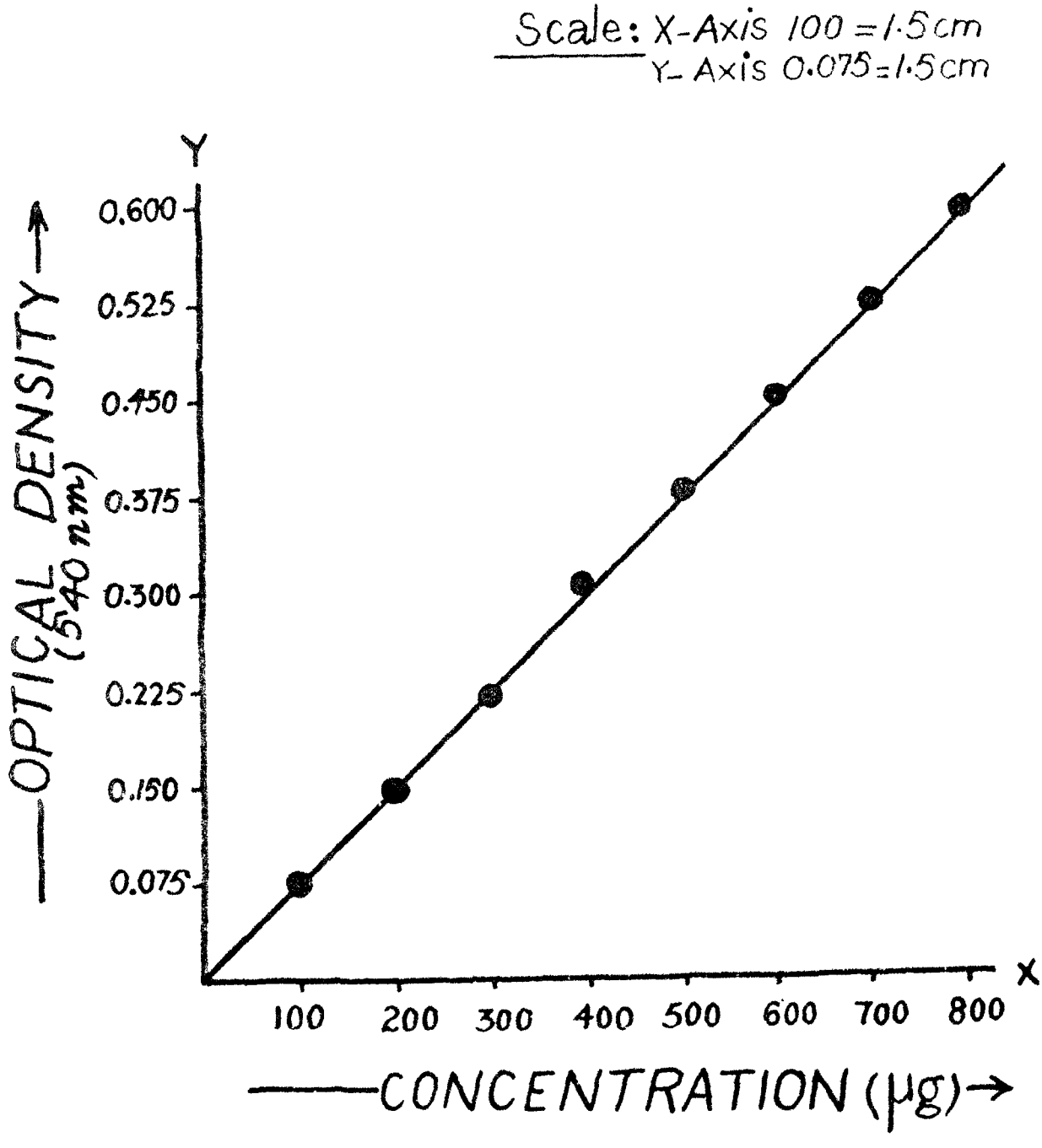


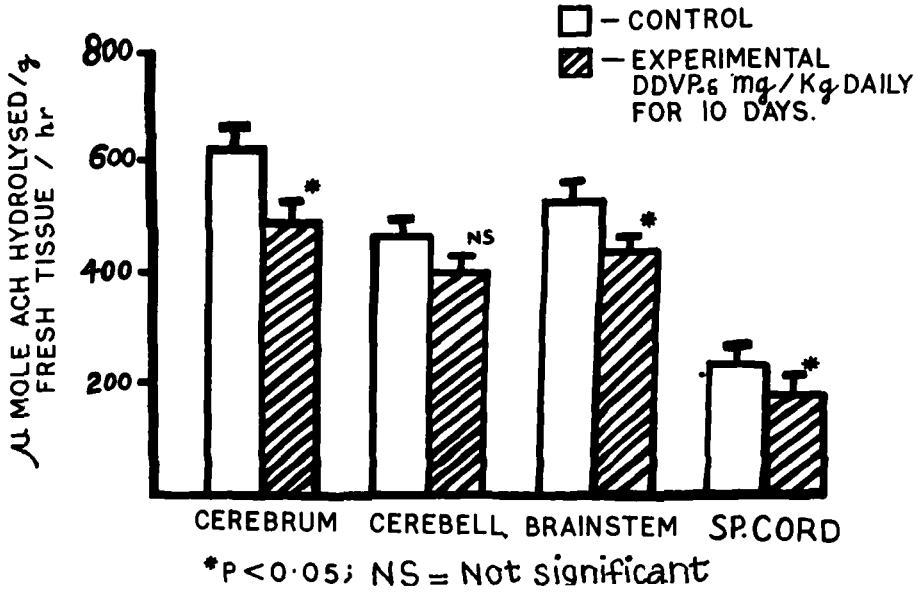
FIG: 10 *Standard curve of* ACETYLCHOLINE

Explanation of Figures:

Fig. 11 : Effects of organophorphate perticide-dichlorvos (0.6 mg/kg body weight i.p. daily for 10 days) on the activity of acetylcholinesterase in different regions of the rat brain and spinal cord. Values represent Mean ± S.E. of 8-10 rats per group. * indicates value significantly different from control, $P < 0.05$; N.S. = Not significant.

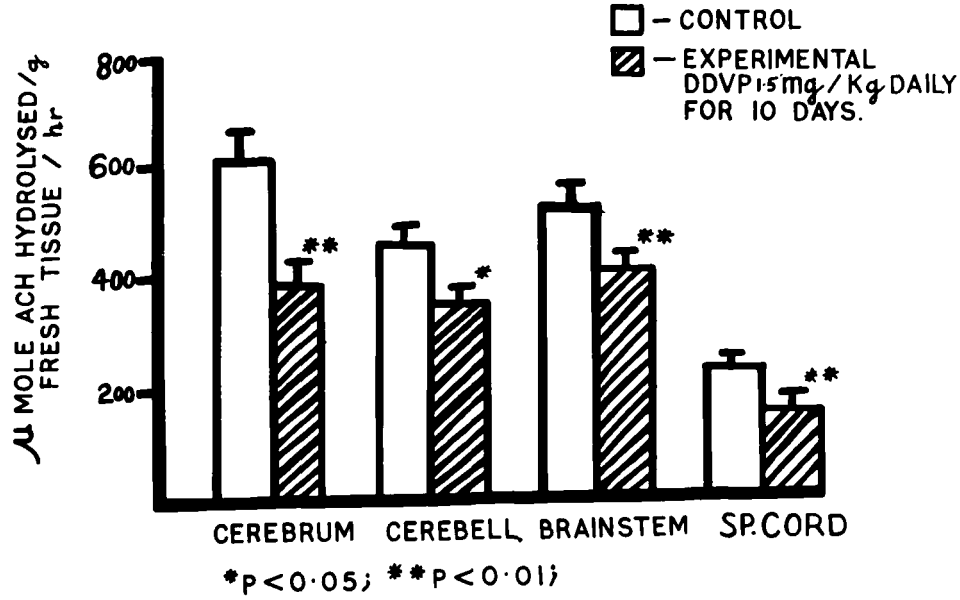
Fig. 12 : Effects of organophorphate perticide-dichlorvos (1.5 mg/kg body weight i.p. daily for 10 days) on the activity of acetylcholinesterase in different regions of the rat brain and spinal cord. Values represent Mean ± S.E. of 8-10 rats per group. * indicates value significantly different from control, $P < 0.05$; ** $P < 0.01$.

ACETYLE CHOLINESTERASE



*P<0·05; NS = Not significant

ACETYLE CHOLINESTERASE



*P < 0·05; **P < 0·01;

11

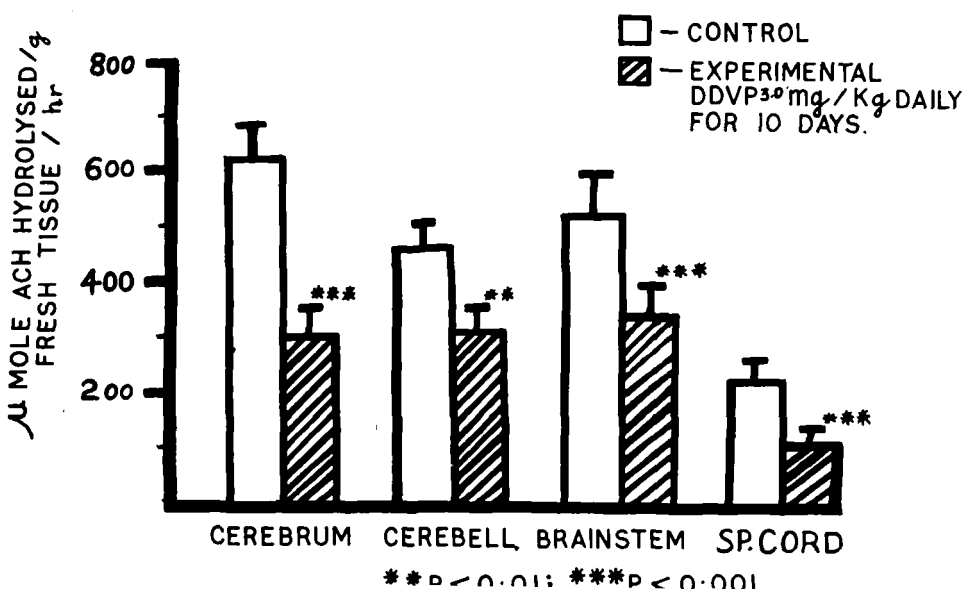
12

Explanation of Figures:

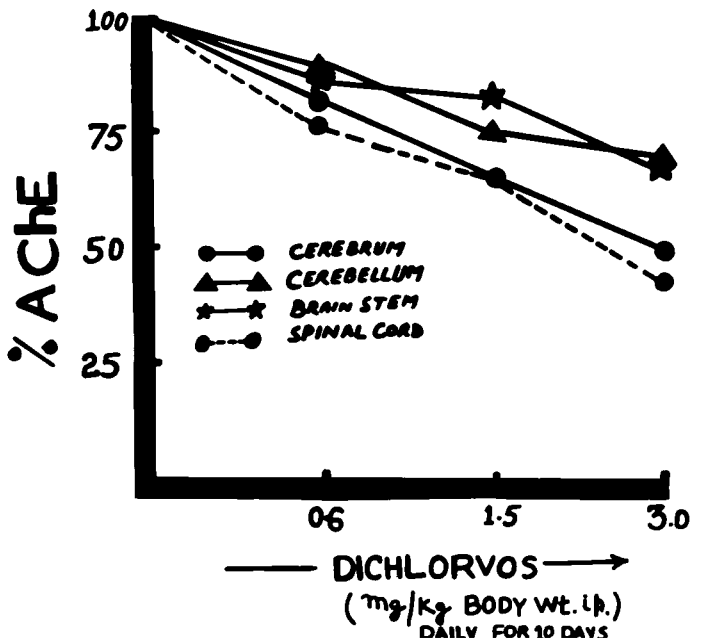
Fig. 13 : Effects of dichlorvos (3.0 mg/kg body weight i.p. daily for 10 days) on the activity of acetylcholinesterase in different regions of the rat brain and spinal cord. Values represent Mean ± S.E. of 8-10 rats per group. ** $P < 0.01$; *** $P < 0.001$.

Fig. 14 : Dose response curve of acetylcholinesterase after the administration of three different doses (0.6, 1.5 and 3.0 mg/kg body weight i.p. daily for 10 days) of dichlorvos. Activity of AChE expressed as μ moles acetylcholine hydrolysed/g fresh tissue/hr.

ACETYLE CHOLINESTERASE



DOSE RESPONSE CURVE



13

14

Scale: X-Axis 0.05 = 1cm
Y-Axis 0.005 = 1cm

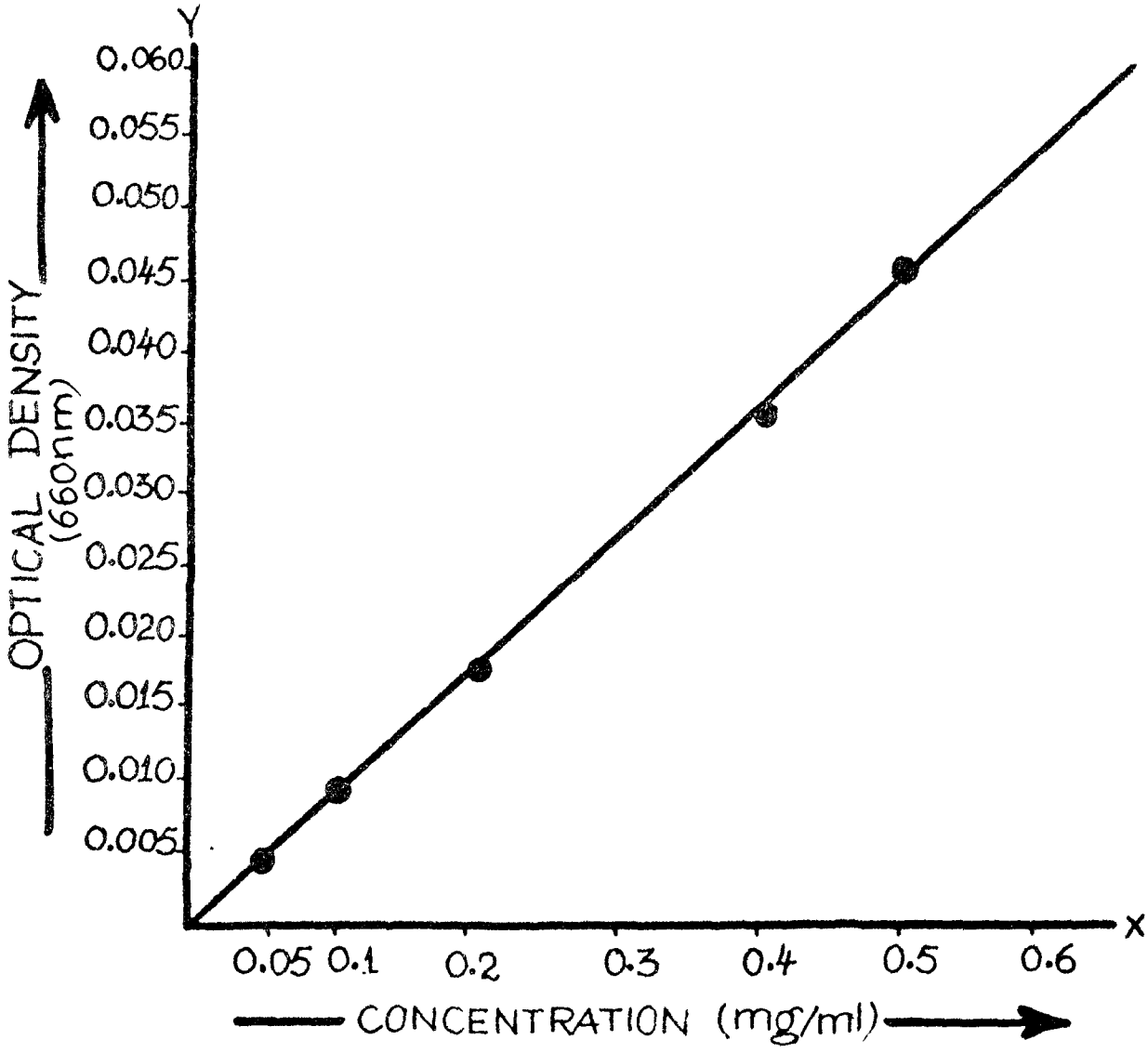


Fig. 15 Standard curve of GABA (Y- Aminobutyric Acid)

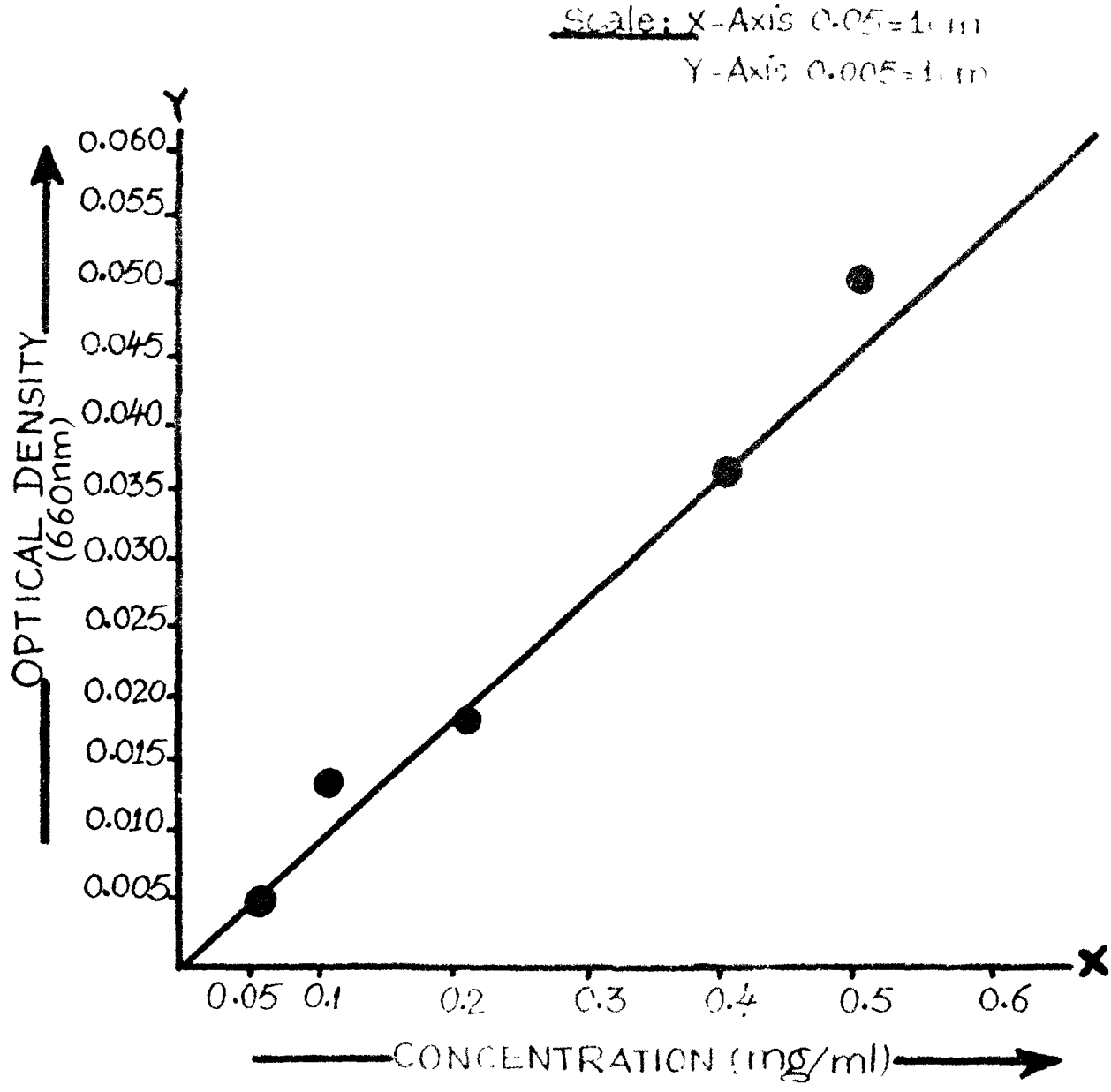


Fig. 16 Standard curve of lysine

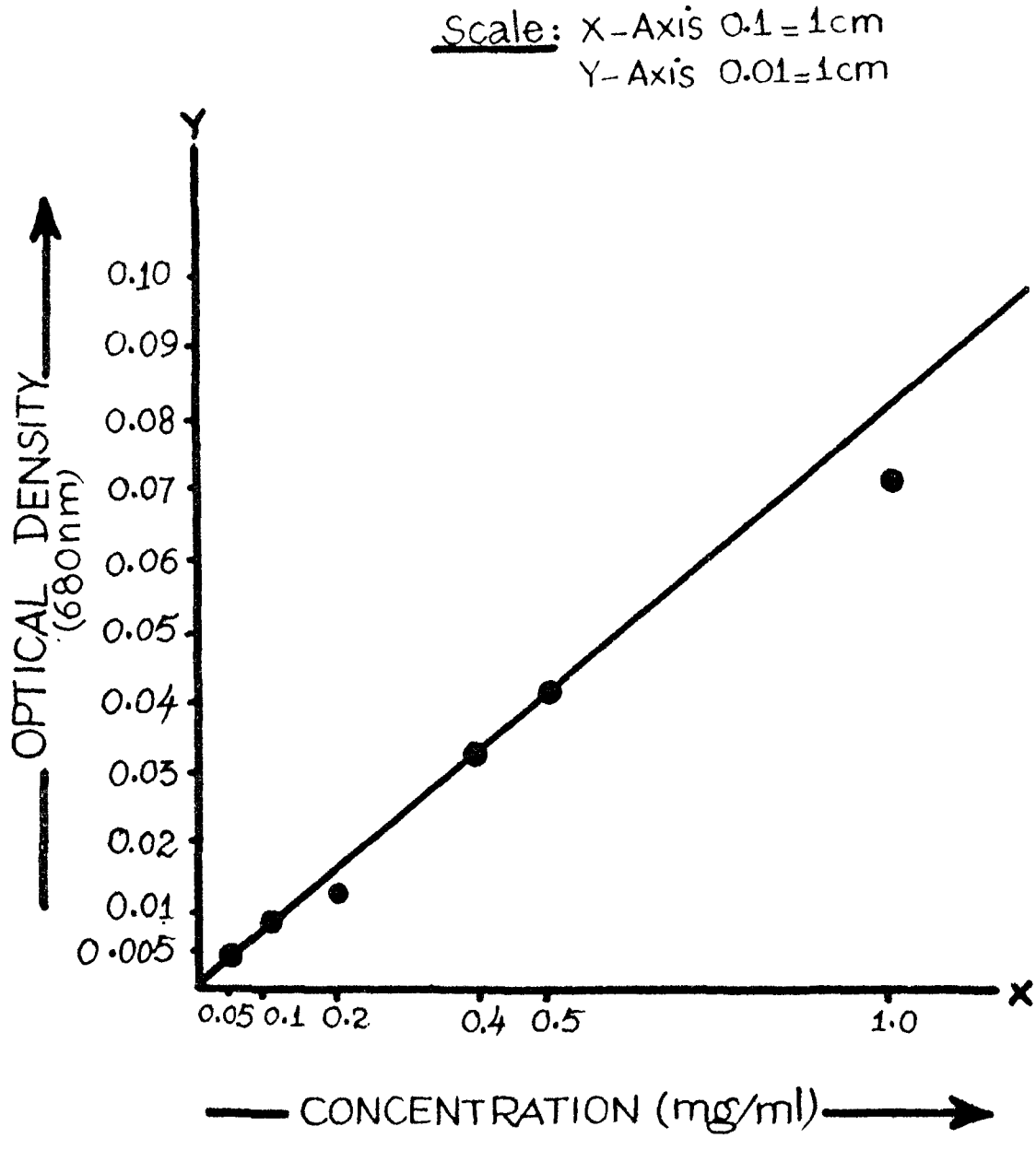


FIG.17 Standard curve of Taurine

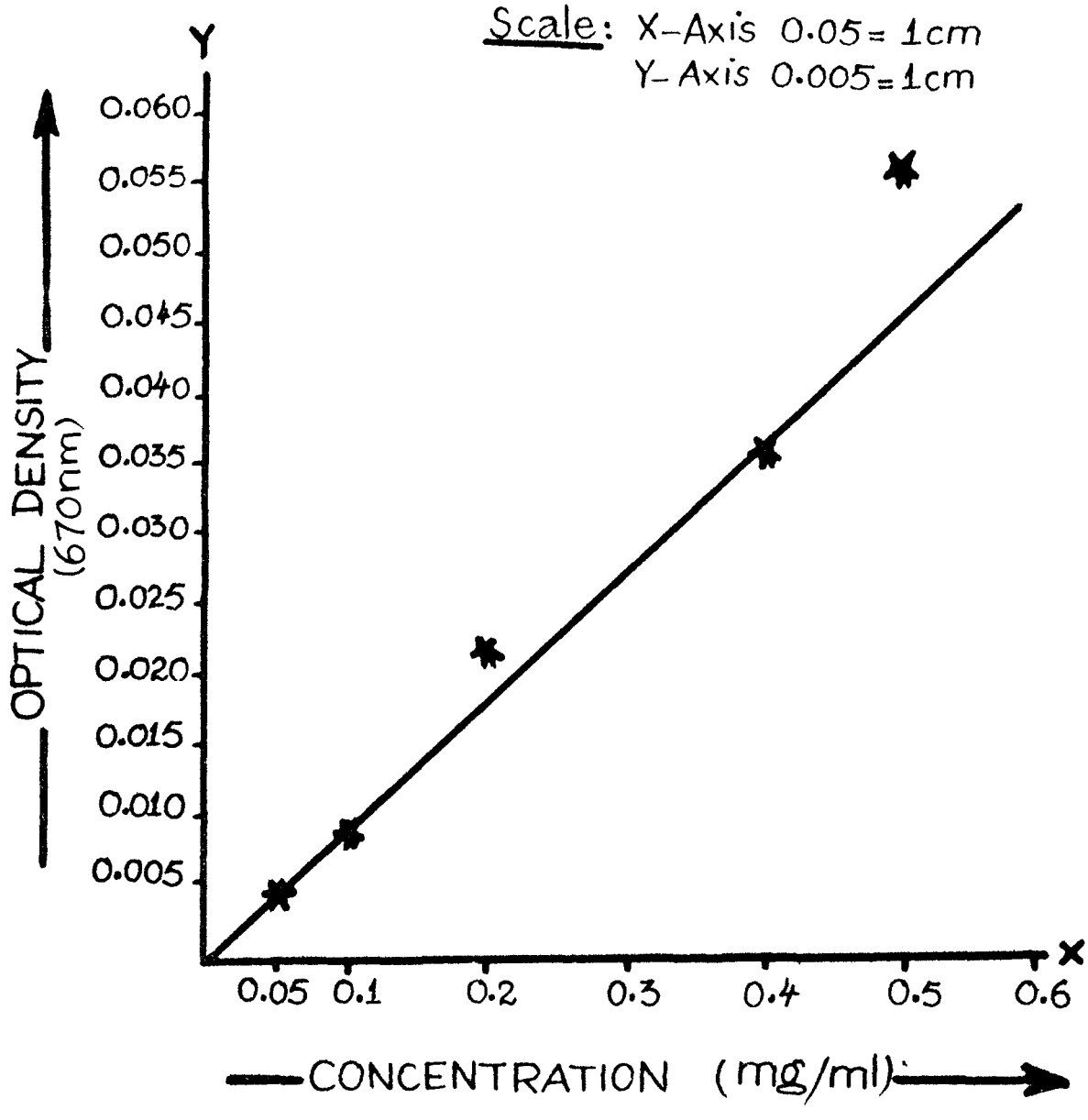


Fig. 18 Standard curve of Aspartic Acid

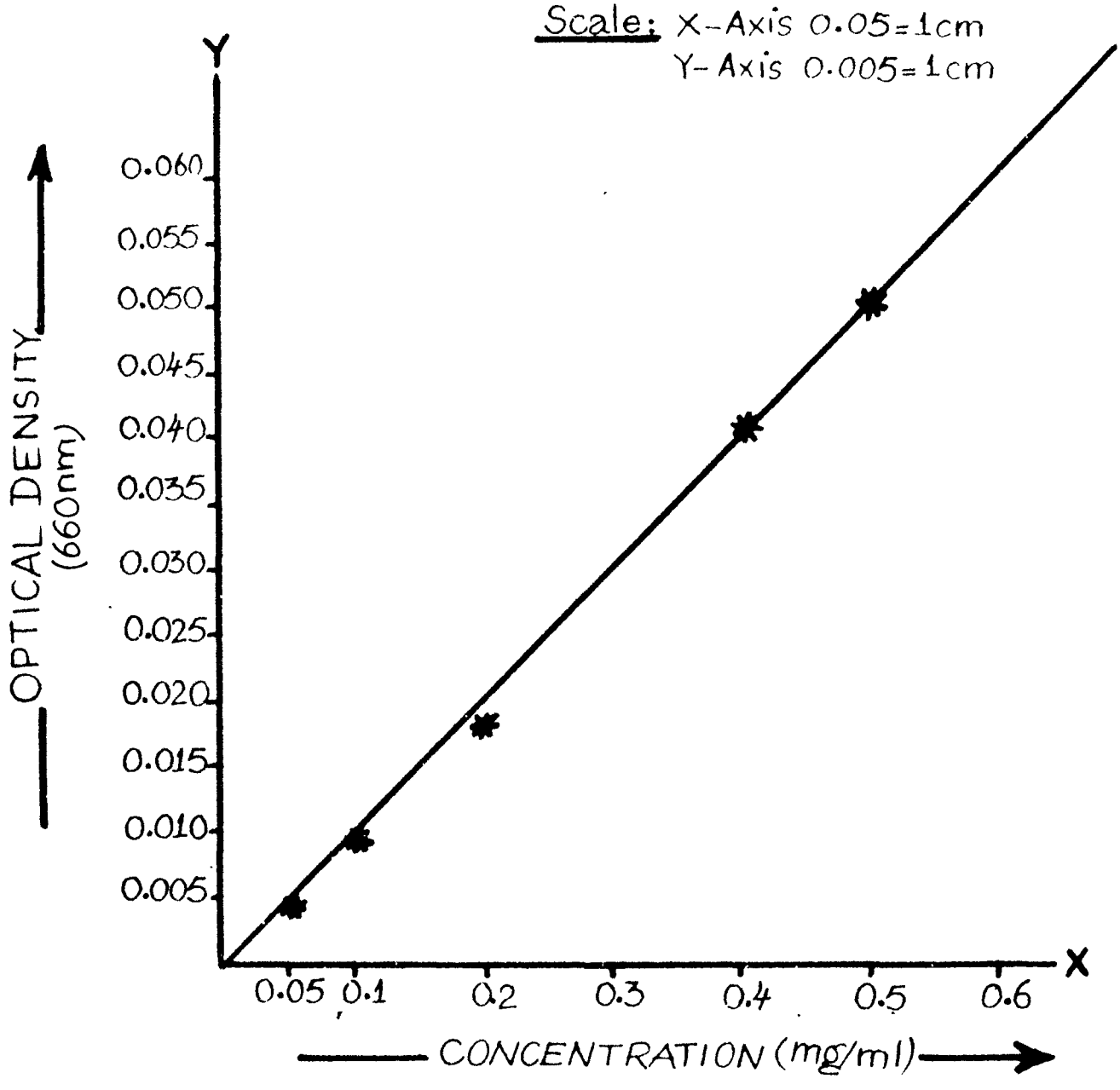


Fig.12 Standard Curve of Serine

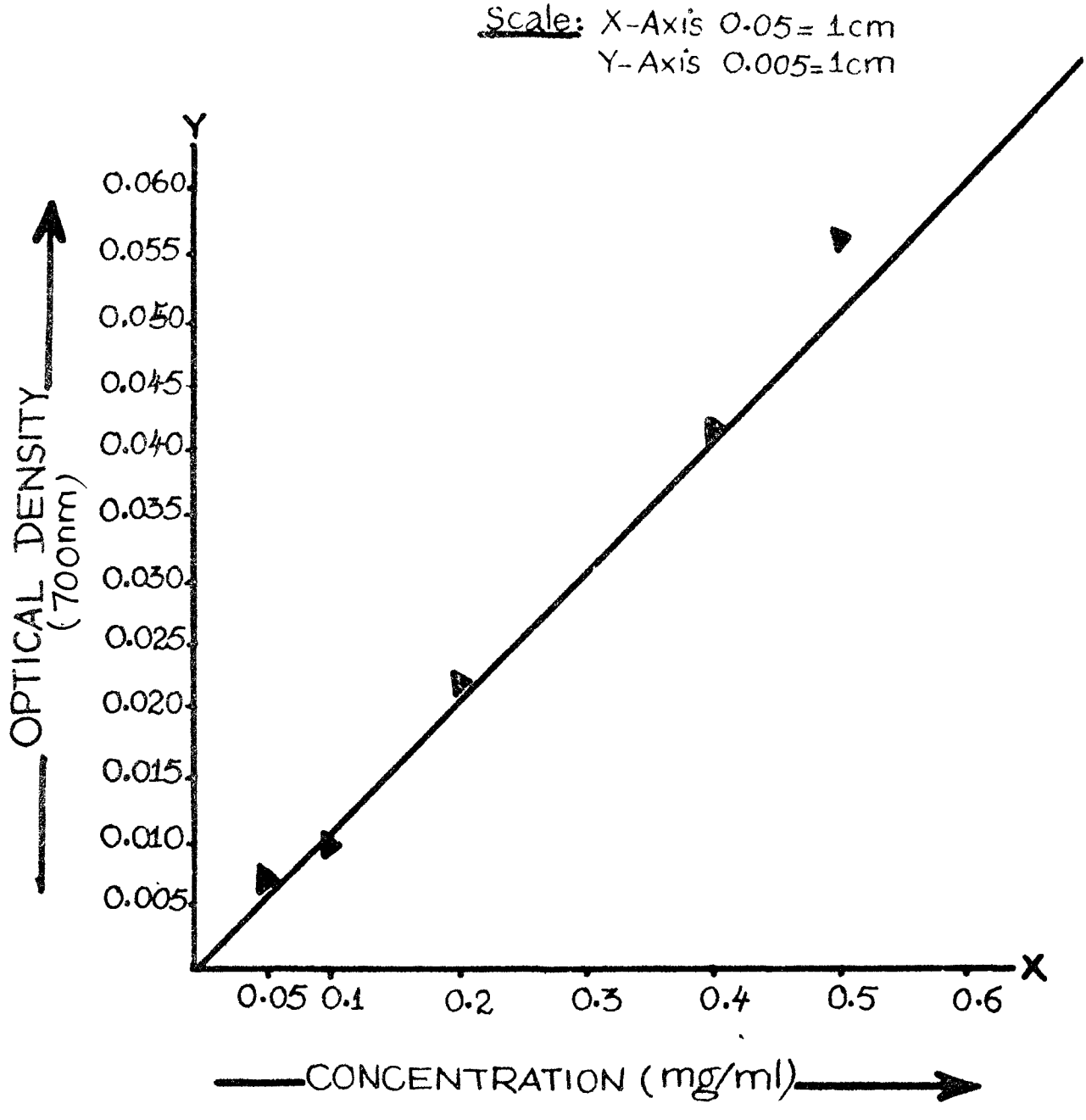


Fig. 20 Standard Curve of glycine

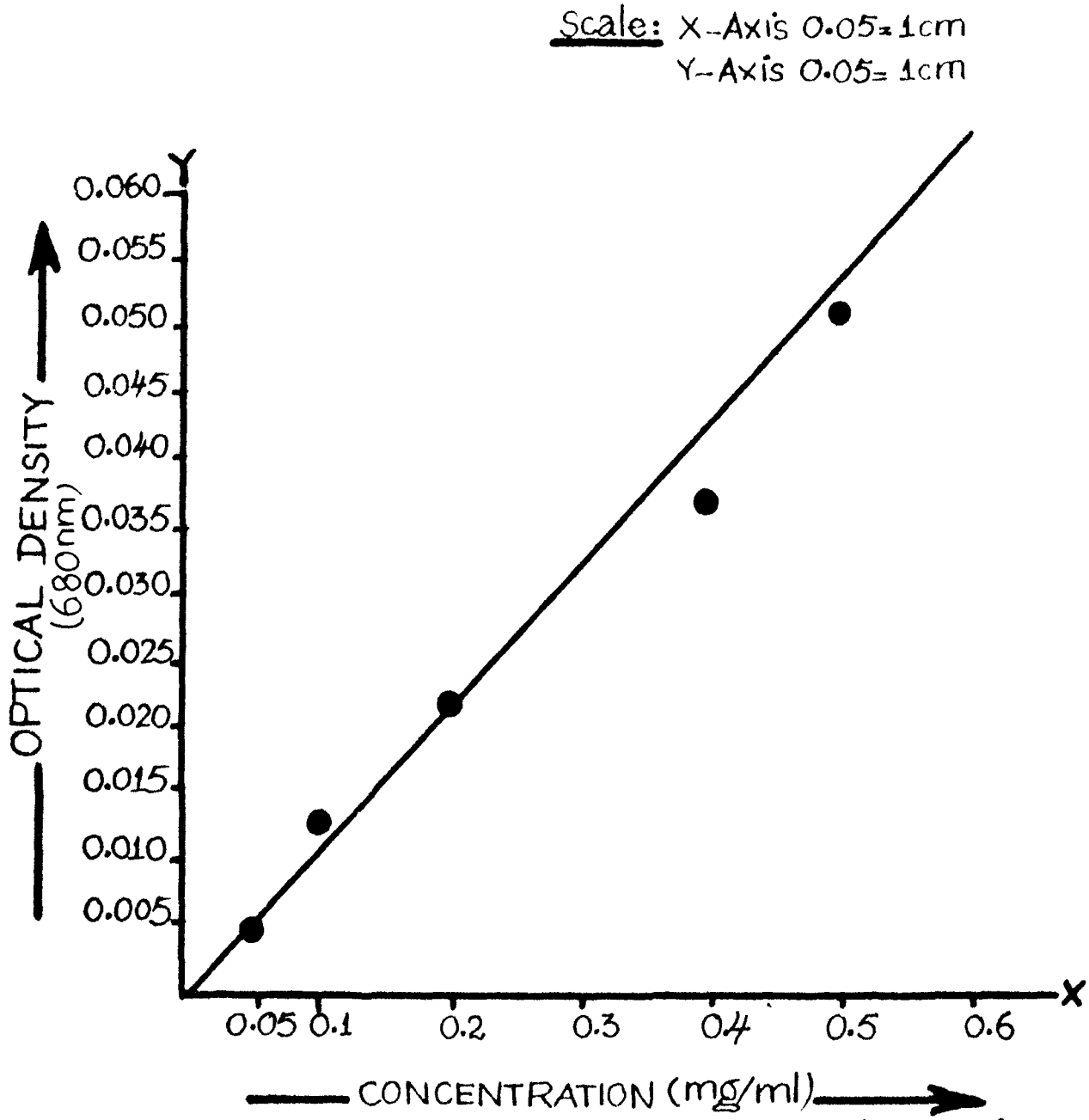
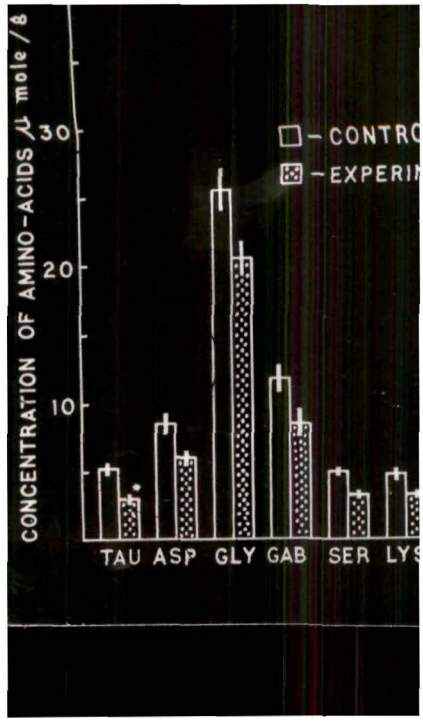


Fig. 21 Standard curve of Phenyl Alanine

Explanation of Figures:

Fig. 22 : The effect of Organophosphate pesticide-dichlorvos (3 mg/kg body weight i.p. daily for 15 days) on the levels of amino acid neurotransmitters in the cerebral hemisphere. Values represent Mean ± S.E. of 10 rats per group. * indicates value significantly different from control, $P < 0.05$.

Fig. 23 : The effect of Organophosphate-dichlorvos (3 mg/kg body weight i.p. daily for 15 days) on the levels of amino acid neurotransmitters in the cerebellum. Values represent Mean ± S.E. of rats per group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.


CONCENTRATION OF AMINO-ACIDS μ mole/g
30
20
10
□ - CONTRO
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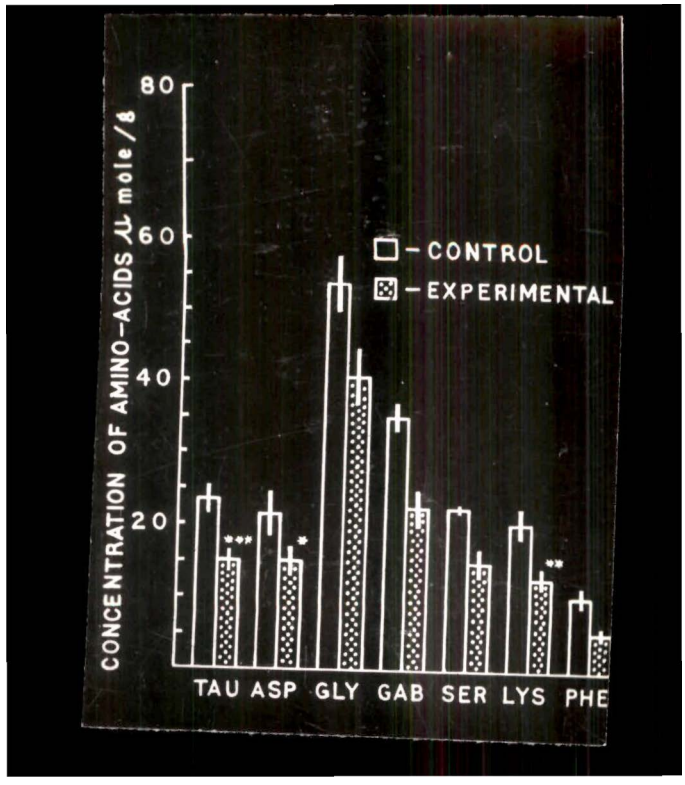
TAU ASP GLY GAB SER LYS


80
60
40
20
CONCENTRATION OF AMINO-ACIDS μ mole/g
□ - CONTROL
▨ - EXPERIMENTAL
**
*
**
TAU ASP GLY GAB SER LYS PHE

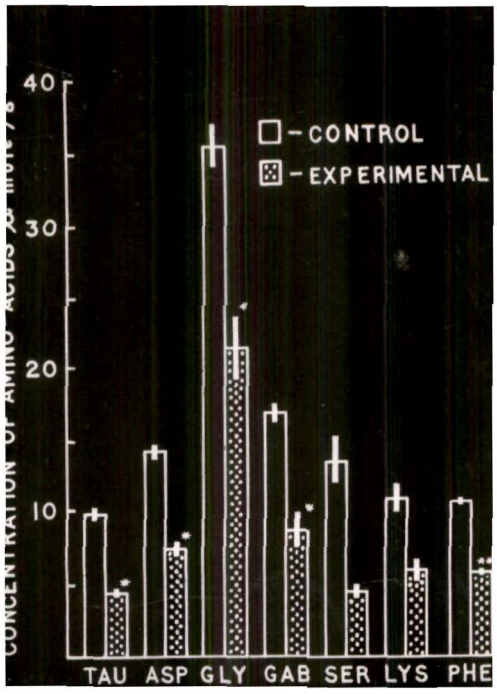
22

23

Explanation of Figures:

Fig. 24 : The effect of Organophorphate-dichlorvos (3 mg/kg body weight i.p. daily for 15 days) on the levels of amino acid neurotransmitters in the brain stem. Values represent Mean ± S.E. of 10 rats per group. * indicate value significantly different from control, $P < 0.05$; *** $P < 0.001$.

Fig. 25 : The effect of Organophorphate-dichlorvos (3 mg/kg body weight i.p. daily for 15 days) on the levels of amino acid neurotransmitters in the spinal cord. Values represent Mean ± S.E. of 10 rats per group. * $P < 0.05$; ** $P < 0.01$.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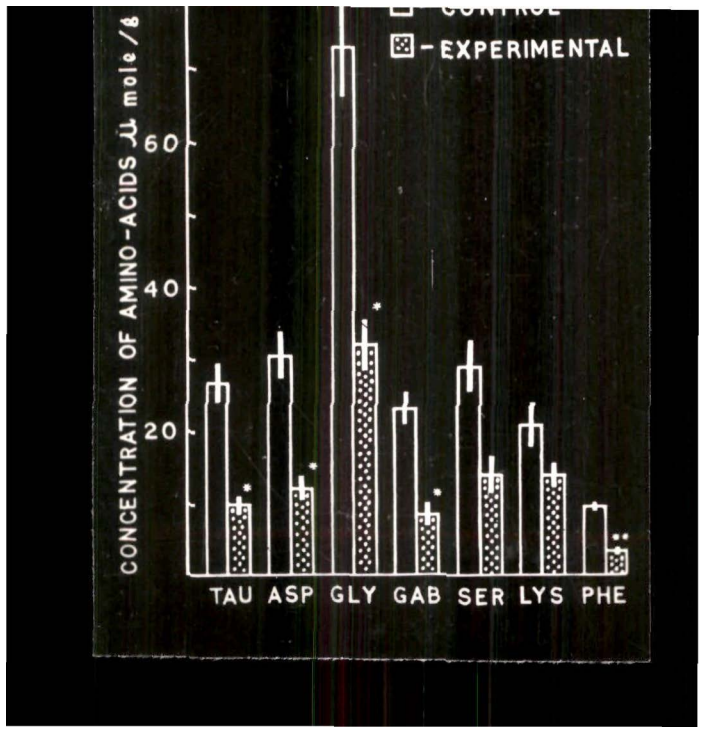

EXPERIMENTAL
CONCENTRATION OF AMINO-ACIDS μ mole/g
60
40
20
TAU ASP GLY GAB SER LYS PHE

dichlorvos on seven amino acids in cerebral hemisphere, cerebellum, brainstem and cervical spinal cord. Statistically significant lowering of taurine was observed in all the regions examined by us. Most significant ($P < 0.001$) decrement was, however, detected in the cerebellum (Fig. 23). The concentration of glycine and GABA was significantly reduce ($P < 0.05$) only in the brain stem and spinal cord (Figs. 24 & 25). Whereas the concentration of aspartic acid showed significant reduction ($P < 0.05$) in the cerebellum, brain stem and spinal cord (Figs. 23-25), the lowering of serine was statistically investigated in all the regions. The concentration of lysine was significantly reduced ($P < 0.1$) in cerebellum only (Fig. 23) and that of the phenyl-alanine (Figs. 24 & 25) in brain stem ($P < 0.001$) and spinal cord ($P < 0.01$).

3.6. Levels of monoamines:

The level of monoamines in different regions of the brain was calculated from the standard curve. Figs. 26-28 show the standard curves of dopamine, NE and 5-HT plotted on different excitation/emission. Table-10 shows the recoveries of dopamine, norepinephrine and 5-hydroxytryptamine. All the three monoamines estimated in the present experiment showed similar pattern. Dopamine was significantly decreased on the fifth, seventh and tenth day in the cerebellum and brain stem, whereas in the cerebral hemisphere the significant decrement was observed only on the seventh day (Table-11). On the tenth day there was almost no change in the amine concentration in the cerebral hemisphere and cerebellum but there was recovery of 13% in the brain stem although it was not statistically significant as

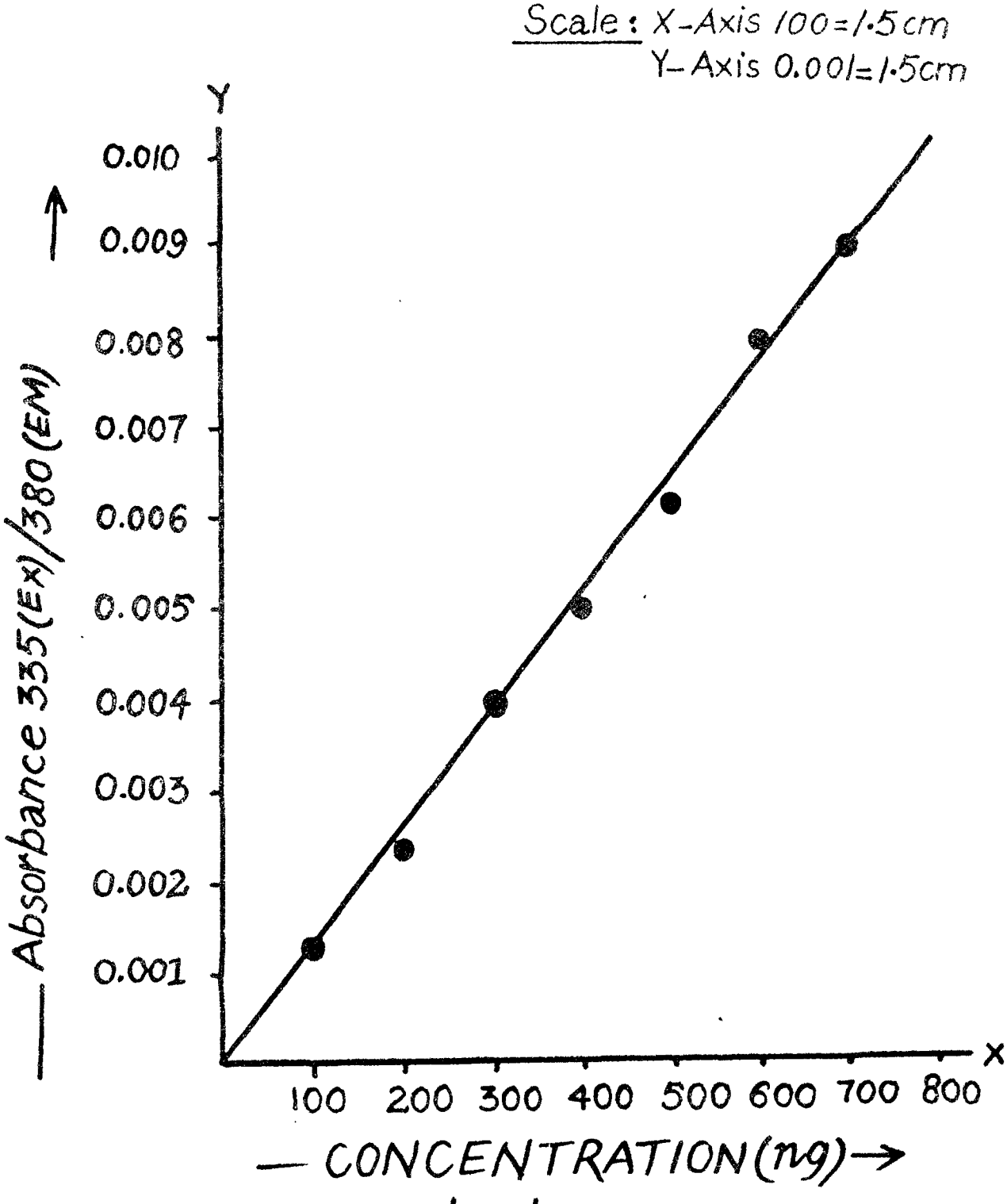


FIG: 26 Standard curve of DOPAMINE

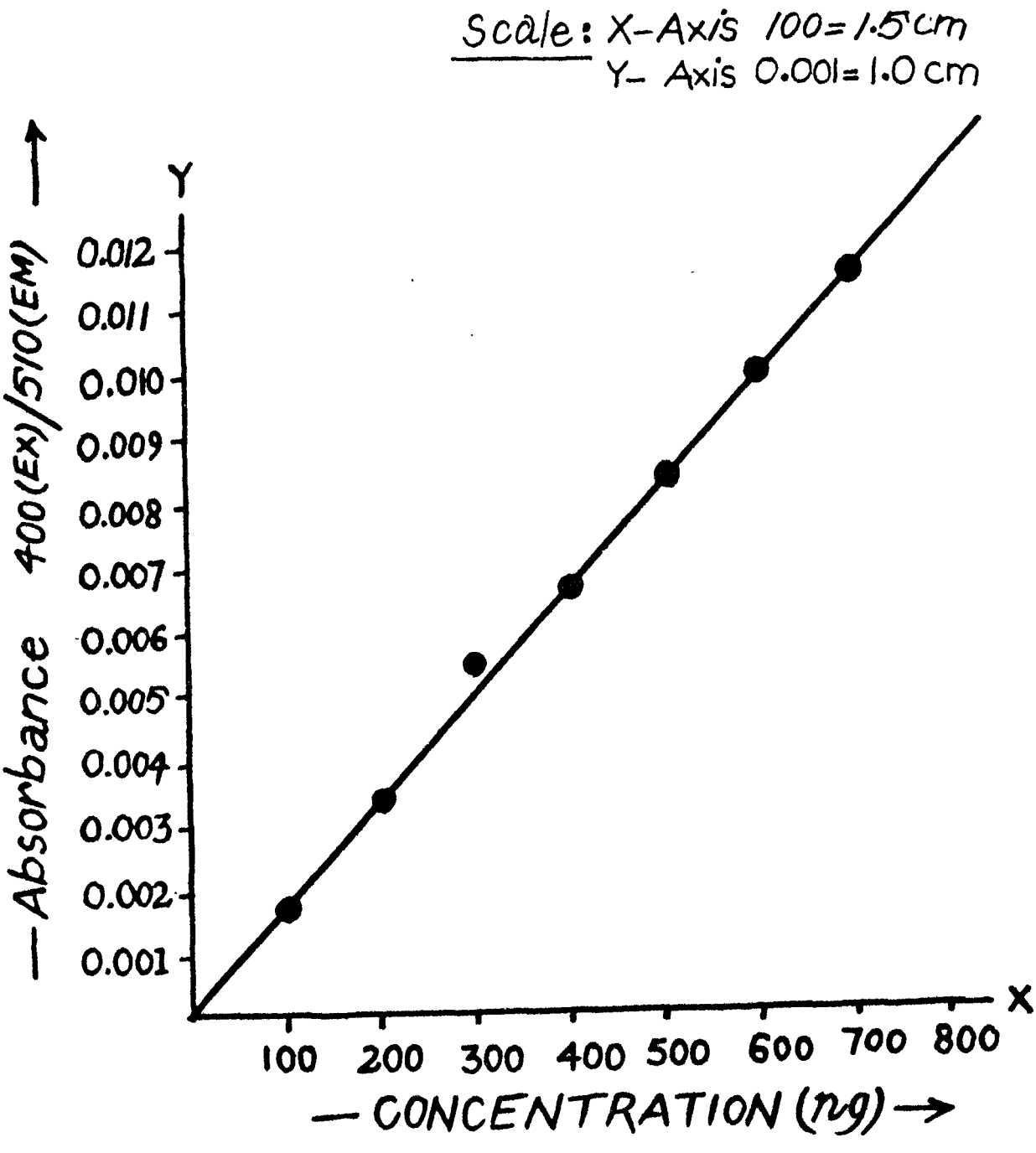


FIG :27 Standard curve of NOREPINEPHRINE

Scale: X-Axis 100=1·5cm
Y- Axis 0·001=1·5cm

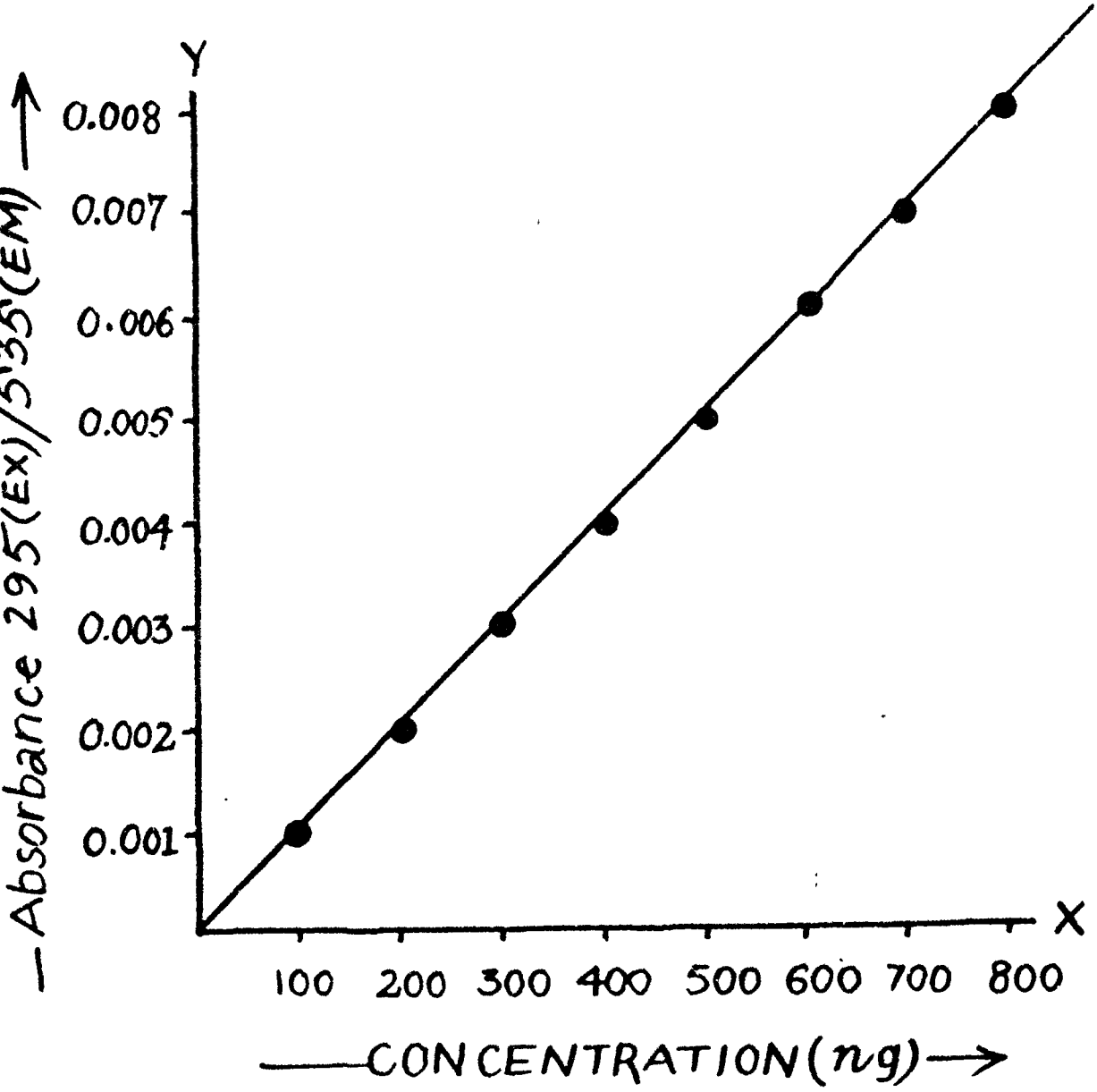


FIG 28: Standard curve of 5-HYDROXY-TRYPTAMINE

Table - 10: Representative recoveries of dopamine, norepinephrine and 5-HT.

COMPOUND	AMOUNT TAKEN ng	AMOUNT RECOVERED ng	% RECOVERY
Dopamine	200	144* ± 3	72
Norepinephrine	100	85* ± 7	85
5-HT	200	156* ± 8	78

* Mean ± S.E. is based upon 10 samples.

compared with its value on the seventh day (Table-11). However, there was no significant change in the level of dopamine in the spinal cord. Norepinephrine was significantly reduced ($P < 0.01$) in the cerebral hemisphere on the seventh day and there was no recovery on the tenth day (Table-12). Levels of norepinephrine were not significantly change in the cerebellum, brain stem and spinal cord. 5-Hydroxytryptamine was significantly reduced in the cerebral hemisphere on seventh and tenth day ($P < 0.05$) and in brain stem on fifth ($P < 0.05$), and tenth ($P < 0.01$) day. The levels of 5-HT were, however, significantly increased ($P < 0.001$) in the spinal cord on the tenth day as compared with the values of control (Table-13).

Table - 11: Level of dopamine in different regions of the rat brain and spinal cord expressed as µg/g fresh tissue (Mean ± S.E.) after the administration of dichlorvos (3 mg/kg body weight, i.p. daily).

BRAIN REGIONS	CONTROL N=15	EXPERIMENTAL 3rd day N=6	% CHANGE	EXPERIMENTAL 5th day N=6	% CHANGE	EXPERIMENTAL 7th day N=10	% CHANGE	EXPERIMENTAL 10th day N=10	% CHANGE
CEREBRAL HEMISPHERE	0.430 ± 0.040	0.400 ± 0.039	- 7	0.370 ± 0.036	-14	0.285* ± 0.037	-34	0.291* ± 0.034	-32
CEREBELLUM	0.292 ± 0.036	0.256 ± 0.032	-12	0.210* ± 0.029	-28	0.195* ± 0.026	-33	0.183* ± 0.021	-37
BRAIN STEM	0.357 ± 0.047	0.345 ± 0.045	- 3	0.260* ± 0.034	-27	0.178** ± 0.018	-50	0.224* ± 0.029	-37
SPINAL CORD	0.138 ± 0.021	0.127 ± 0.011	- 8	0.123 ± 0.011	-11	0.120 ± 0.011	-13	0.157 ± 0.014	+13

N = Number of animals used in each experiment.

* $P < 0.05$; ** $P < 0.01$.

Table - 12: Level of Norepinephrine in different regions of the rat brain and spinal cord expressed as /ug/g fresh weight (Mean ± S.E.) - after the administration of Dichlorvos (3 mg/kg body weight, i.p. daily).

BRAIN REGION AND SPINAL CORD	CONTROL N=15	EXPERIMENTAL 3rd day N=6	% CHANGE	EXPERIMENTAL 5th day N=6	% CHANGE	EXPERIMENTAL 7th day N=10	% CHANGE	EXPERIMENTAL 10th day N=10	% CHANGE
CEREBRAL HEMISPHERE	1.626 ± 0.099	1.424 ± 0.101	-12	1.236 ± 0.096	-24	0.848* ± 0.091	-48	0.818* ± 0.085	-50
CEREBELLUM	1.257 ± 0.204	1.268 ± 0.216	+ 1	1.040 ± 0.121	-17	0.979 ± 0.016	-22	0.965 ± 0.014	-23
BRAIN STEM	1.022 ± 0.127	0.955 ± 0.104	- 7	0.868 ± 0.096	-15	0.761 ± 0.081	-25	0.801 ± 0.095	-21
SPINAL CORD	0.571 ± 0.086	0.561 ± 0.088	- 2	0.506 ± 0.076	-12	0.469 ± 0.071	-18	0.431 ± 0.060	-24

N = Number of animals used in each experiment.

* $P < 0.01$.

Table - 13: Level of 5-Hydroxytryptamine in different regions of the rat brain and spinal cord expressed as µg/g fresh tissue (Mean ± S.E.) after the administration of Dichlorvos (3 mg/kg body weight, i.p. daily).

BRAIN REGION AND SPINAL CORD	CONTROL N=15	EXPERIMENTAL 3rd day N=6	% CHANGE	EXPERIMENTAL 5th day N=6	% CHANGE	EXPERIMENTAL 7th day N=10	% CHANGE	EXPERIMENTAL 10th day N=10	% CHANGE
CEREBRAL HEMISPHERE	0.446 ± 0.057	0.389 ± 0.046	-13	0.360 ± 0.040	-19	0.295* ± 0.039	-34	0.281* ± 0.035	-36
CEREBELLUM	0.376 ± 0.063	0.370 ± 0.065	- 2	0.331 ± 0.051	-12	0.312 ± 0.041	-17	0.291 ± 0.032	-22
BRAIN STEM	0.403 ± 0.030	0.358 ± 0.029	-11	0.276* ± 0.028	-32	0.209** ± 0.034	-48	0.201** ± 0.026	-50
SPINAL CORD	0.101 ± 0.007	0.112 ± 0.009	+11	0.123 ± 0.013	+21	0.126 ± 0.019	+24	0.163*** ± 0.008	+60

N = Number of animals used in each experiment.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3.7. Rate of lipid peroxidation:

The rate of lipid-peroxidation expressed as moles of malonaldehyde formed/30 minutes in different regions of the rat brain after the administration of three doses of organophosphate-dichlorvos is represented in Table-14. At the dose of 0.6 mg/kg the rate of lipid-peroxidation was significantly increased in cerebellum (+32%) and brain stem (+53%). However, no significant change (+11%) was observed in the cerebral hemisphere. When the dose of organophosphate was increased (1.5 mg/kg) the rate of lipid-peroxidation was also increased from 11 to 47% in cerebral hemisphere, 32 to 43% in cerebellum and 53 to 100% in brain stem. Furthermore, at the dose of 3.0 mg/kg, the rate of lipid-peroxidation was increased significantly ($P < 0.001$) in all the three different regions of the brain when compared with the values of control (Table-14).

3.8. Gross findings of the brain and spinal cord:

The autopsied brain showed occasional congestion of meninges and signs of minimal cerebral oedema. Brain weight of organophosphate-dichlorvos rats did not differ significantly from that of the controls. The ventricular system was bilaterally symmetrical. Coronal sections at various levels also did not reveal any abnormality.

Table - 14: Rate of lipid-peroxidation expressed as moles of malonaldehyde formed/30 minute, in different regions of the rat brain after the administration of different doses of dichlorvos i.p. daily for 10 days (Mean ± S.E.).

DIFFERENT REGION OF RAT BRAIN	CONTROL N=10	EXPERIMENTAL DICHLORVOS 0.6 mg/kg N=10	% CHANGE	P	EXPERIMENTAL DICHLORVOS 1.5 mg/kg N=10	% CHANGE	P	EXPERIMENTAL DICHLORVOS 3.0 mg/kg N=10	% CHANGE	P
CEREBRAL HEMISPHERE	2.482 ± 0.333	2.758 ± 0.479	+11	< 0.2 N.S.	3.648 ± 0.342	+47	< 0.001	4.506 ± 0.350	+81	< 0.001
CEREBELLUM	2.305 ± 0.319	3.036 ± 0.352	+32	< 0.001	3.306 ± 0.142	+43	< 0.001	3.609 ± 0.331	+57	< 0.001
BRAIN STEM	1.430 ± 0.173	2.190 ± 0.173	+53	< 0.001	2.868 ± 0.198	+100	< 0.001	3.277 ± 0.137	+129	< 0.001

N = Number of animals used in each experiment.

P < 0.05 - Significant; P < 0.2 - N.S. = Not significant.

3.9. Light microscopic changes:

Histochemical changes:

(a) **Acetylcholinesterase:** The activity of acetylcholinesterase in the cerebral cortex of the organophosphate-dichlorvos intoxicated rats was found to be significantly diminished (Fig. 29) when compared with the normal control (Fig. 30).

(b) **Cytochrome oxidase:** Enzyme activity of the organophosphate-dichlorvos-intoxicated neurones of the rat cerebrum (Fig. 31) was apparently reduced in comparison with the normal control (Fig. 32).

(c) **Succinic dehydrogenase:** Succinic dehydrogenase activity of the neurones in cerebral cortex of organophosphate-dichlorvos-intoxicated rats was found to be diminished (Fig. 33) when compared with the normal control (Fig. 34).

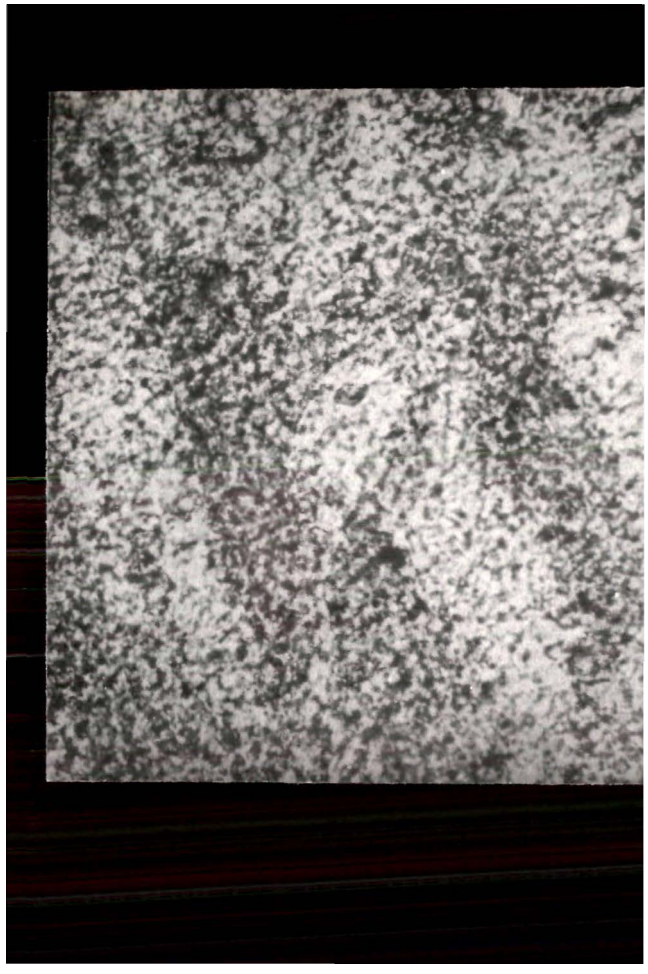
3.10. Electron microscopy:

Spinal cord: The neurons in spinal cord of the control rats exhibited well preserved membranes, Golgi apparatus, mitochondria, the rough endoplasmic reticulum and rosettes of ribosome were interspersed in the cytoplasm (Fig. 35). Occasionally, a few osmophilic bodies were noted in the immediate vicinity of the nucleus (Fig. 36). The neuropil showed well preserved synapses (Fig. 37). In the

Explanation of Figures:

Fig. 29 : The activity of acetylcholinesterase in the cerebral cortex of the organophosphate-dichlorvos intoxicated rat (X 625).

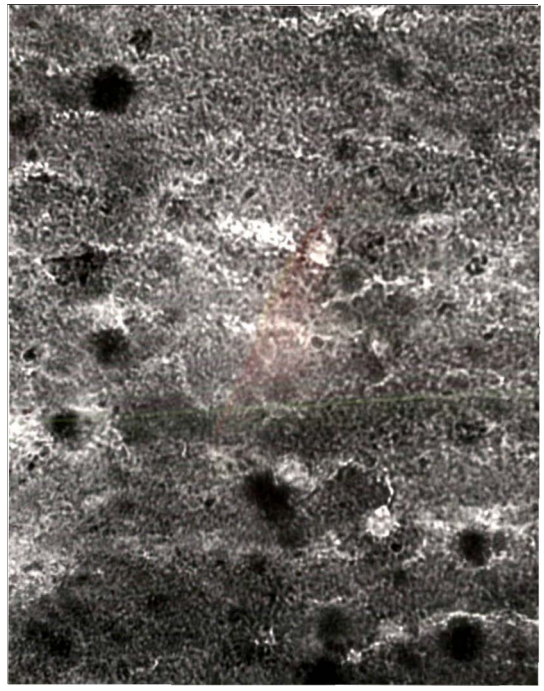
Fig. 30 : Acetylcholinesterase activity of the normal control rat cerebral cortex (X 625).



Explanation of Figures:

Fig. 31 : Activity of cytochrome oxidase of organophosphate-dichlorvos intoxicated rat cerebrum (X 525).

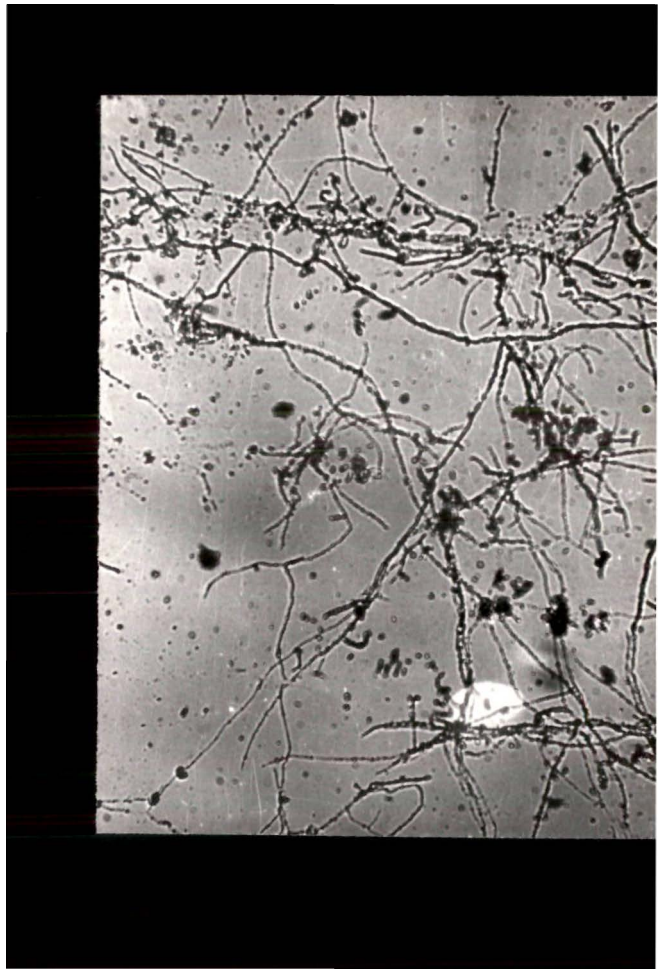
Fig. 32 : Cytochrome oxidase activity of normal control rat cerebrum (X 625).



Explanation of Figures:

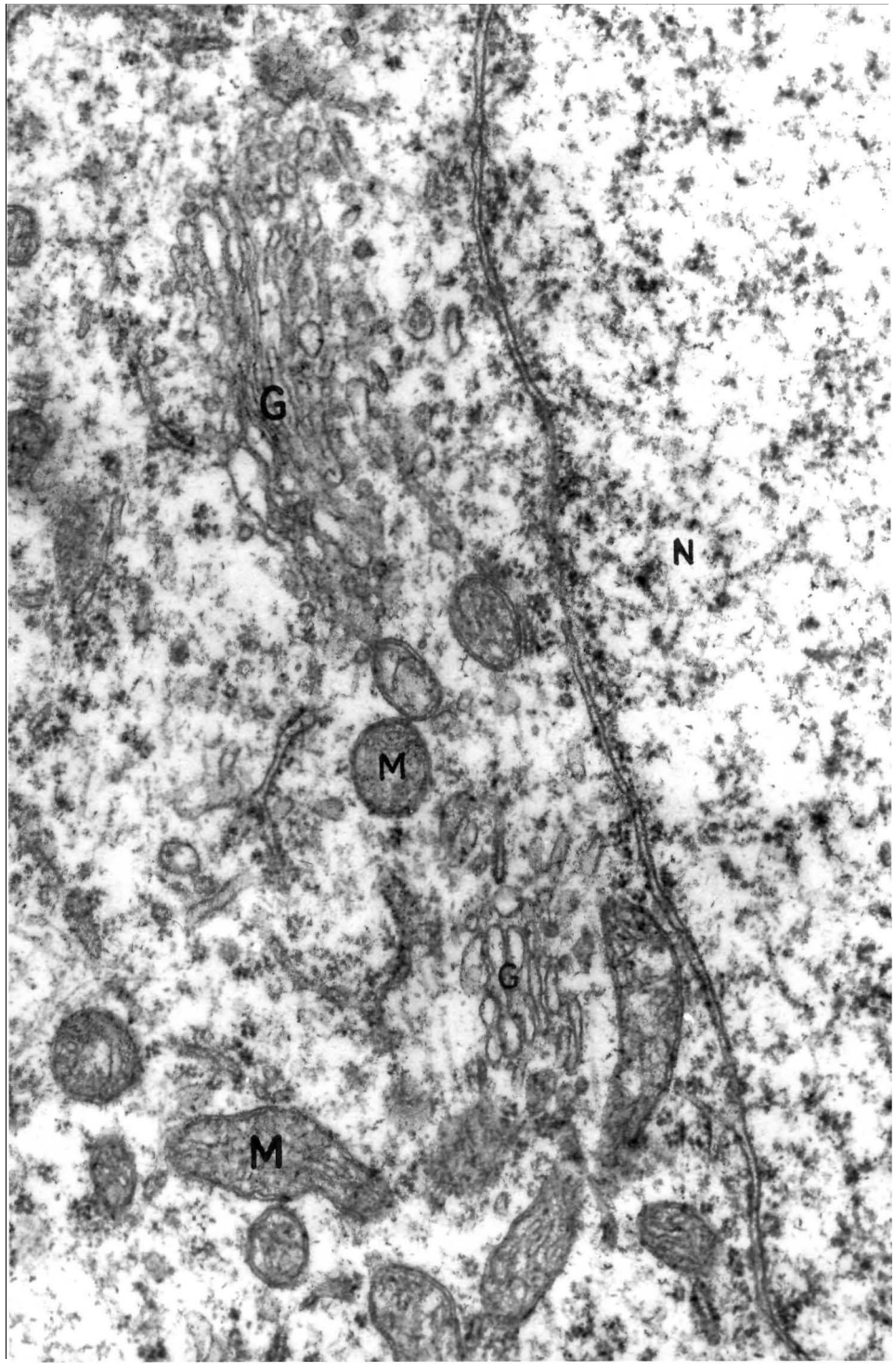
Fig. 33 : Succinic dehydrogenase activity of cerebral cortex of organophosphate-dichlorvos intoxicated rat (X 625).

Fig. 34 : Succinic dehydrogenase activity of the normal control rat cerebral cortex (X 525).



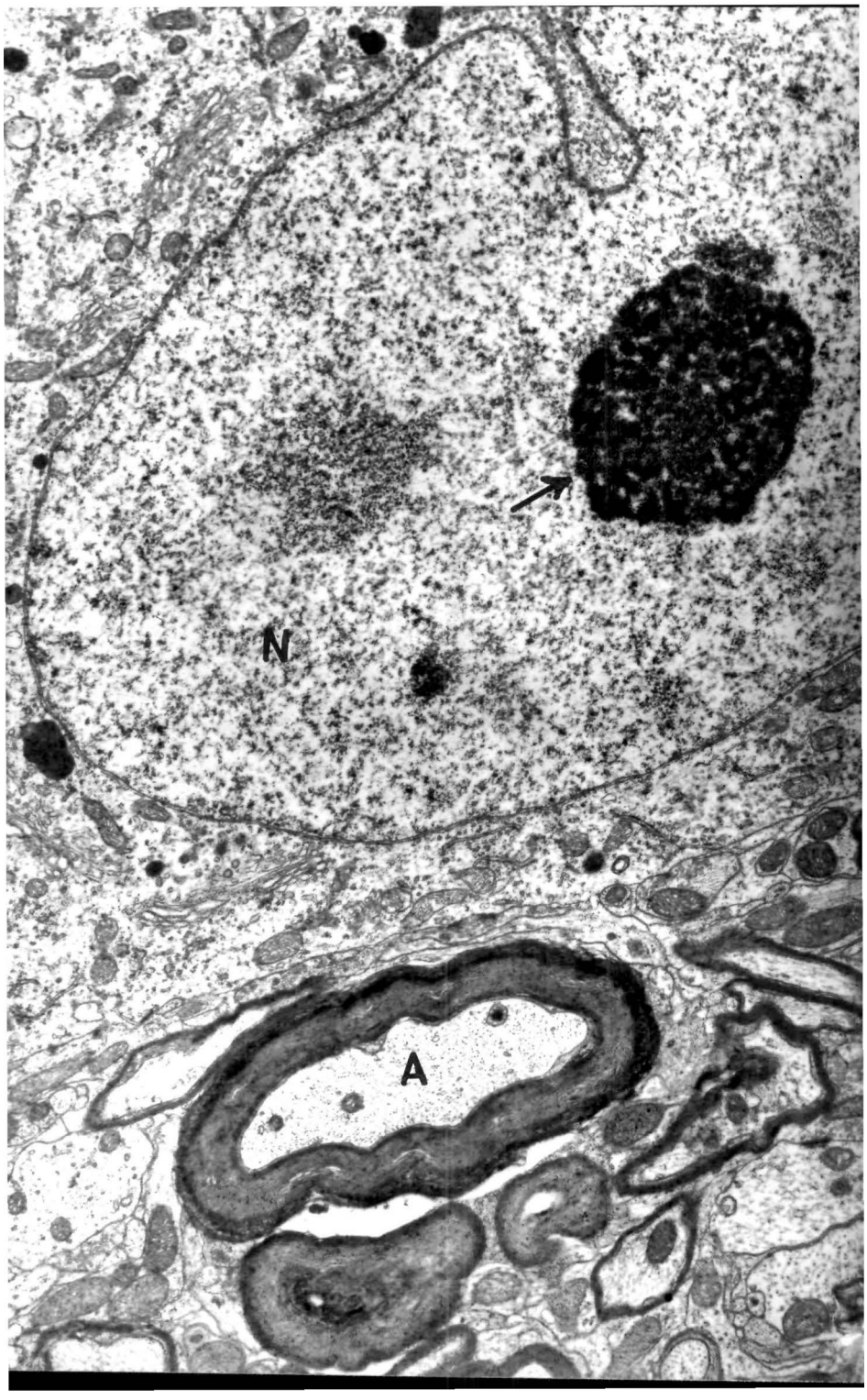
Explanation of Figure:

Fig. 35 : Electron micrograph (EM) of a part of spinal cord neurone of control rat showing well preserved membranes, golgi apparatus and mitochondria (X 36,000).


G
N
M
G
M

Explanation of Figure:

Fig. 36 : Electron micrograph of a part of spinal cord neurone of control rat. A nucleus (N) containing a nucleolus (arrow) is seen with one electron dense body and axon (A) in the vicinity of the nuclear membrane (X 12,000).


N
A

Explanation of Figure:

Fig. 37 : Electron micrograph of neuropil of the control rat spinal cord. Note the normal electron density of the axon (A) and dendrites. Also note the well preserved synapse and mitochondria (X 32,400).

37

experimental group of rat however, increased incidence of cluster of osmophilic bodies resembling lipofuscin granules was detected in the perikaryon (Figs. 39-40). The Golgi apparatus occasionally presented dilated cisternae (Fig. 41).

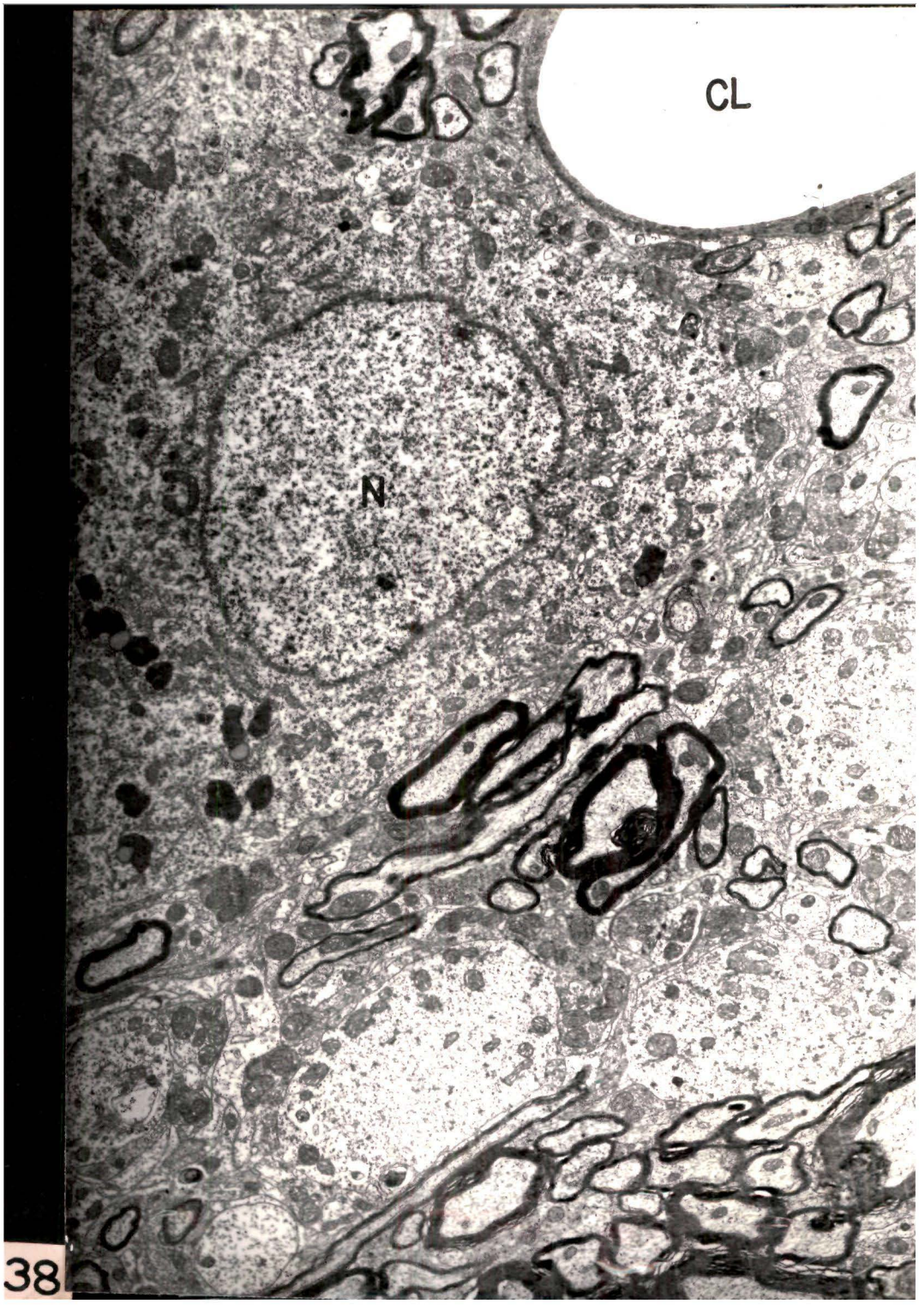
Cerebellum: In the cerebellum well preserved rough endoplasmic reticulum and mitochondria were detected in the experimental rats. The most noticeable finding was the increased incidence of pleomorphic osmophilic bodies exhibiting small electron lucid vacuoles amongst degenerating mitochondrial profiles (Figs. 42-44). Some nuclear profiles showed wavy contour and invagination. There also dilated cisternal of smooth endoplasmic reticulum were apparent (Fig. 45). Oligodendrocytes also contained myelin rests and dilated spaces (Fig. 46).

In the hypothalamus axonal profiles were full of spherical and cylindrical tubular vesicles (Fig. 47). At places the axons were surrounded by electron lucid profiles suggestive of accumulation of fluid in astrocytic processes (oedema) (Fig. 48).

In the hippocampus clumps of osmophilic bodies, presumably lipofuscin, filled with electron dense particle and electron lucid vacuoles were detected in the neuronal perikaryon. It appear that smaller component of dense bodies had fused to form bigger bodies (Fig. 49). Occasionally, satellite oligodendrocytes containing electron dense material in the perikarya were noticeable. The plasma membrane of oligodendrocytes and neuron appeared to be forming tight junction (Fig. 50).

Explanation of Figure:

Fig. 38 : Electron micrograph of a part of the organophosphate-dichlorvor treated rat spinal cord. Note the increased incidence of electron dense bodies in the vicinity of the nucleus (X 7,250).



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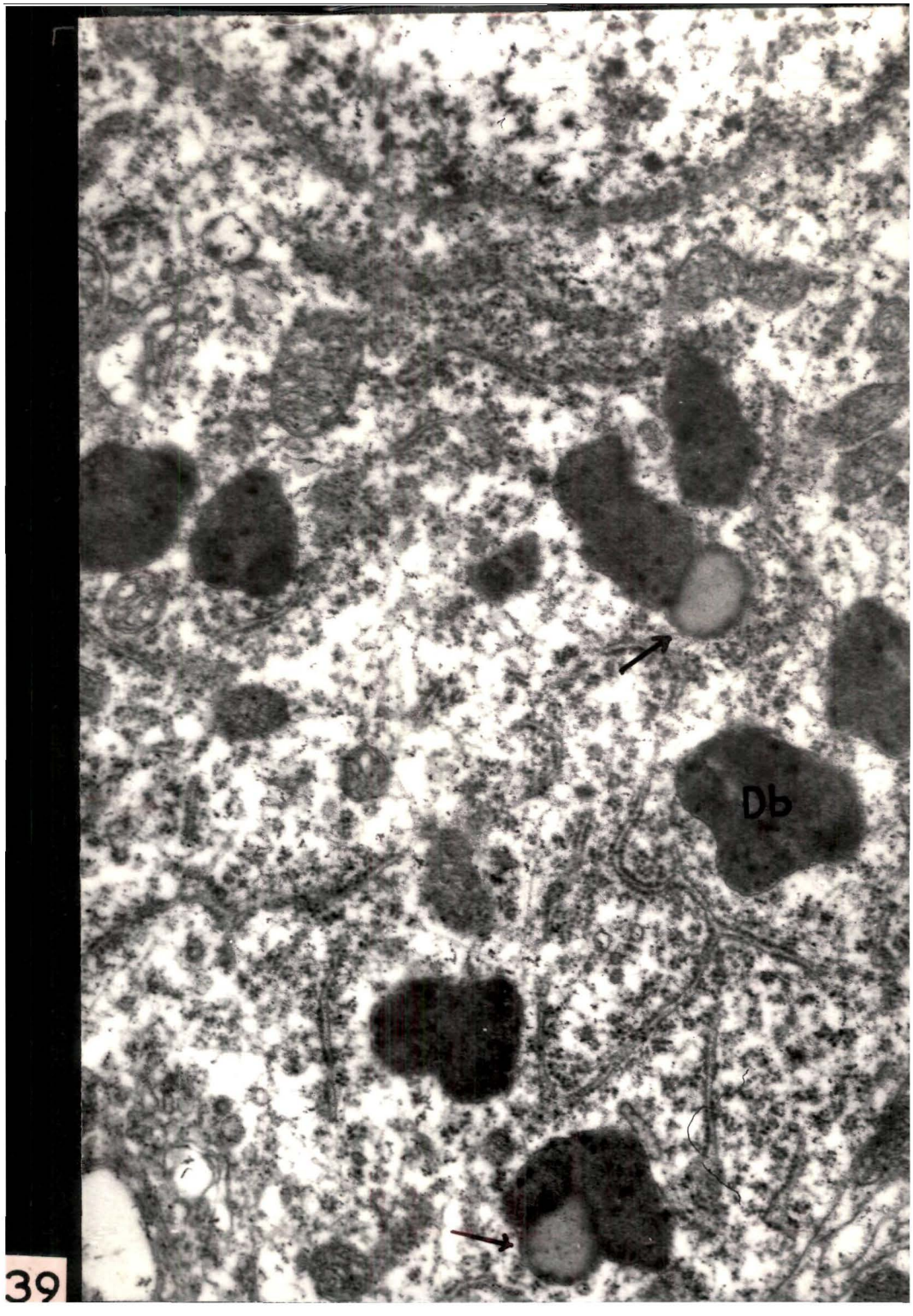
Explanation of Figure:

Fig. 39 : Electron micrograph, higher magnification of Fig. 38. Note the increased incidence of electron-dense bodies, two showing electron lucid vacuoles (arrows) (X 32,400).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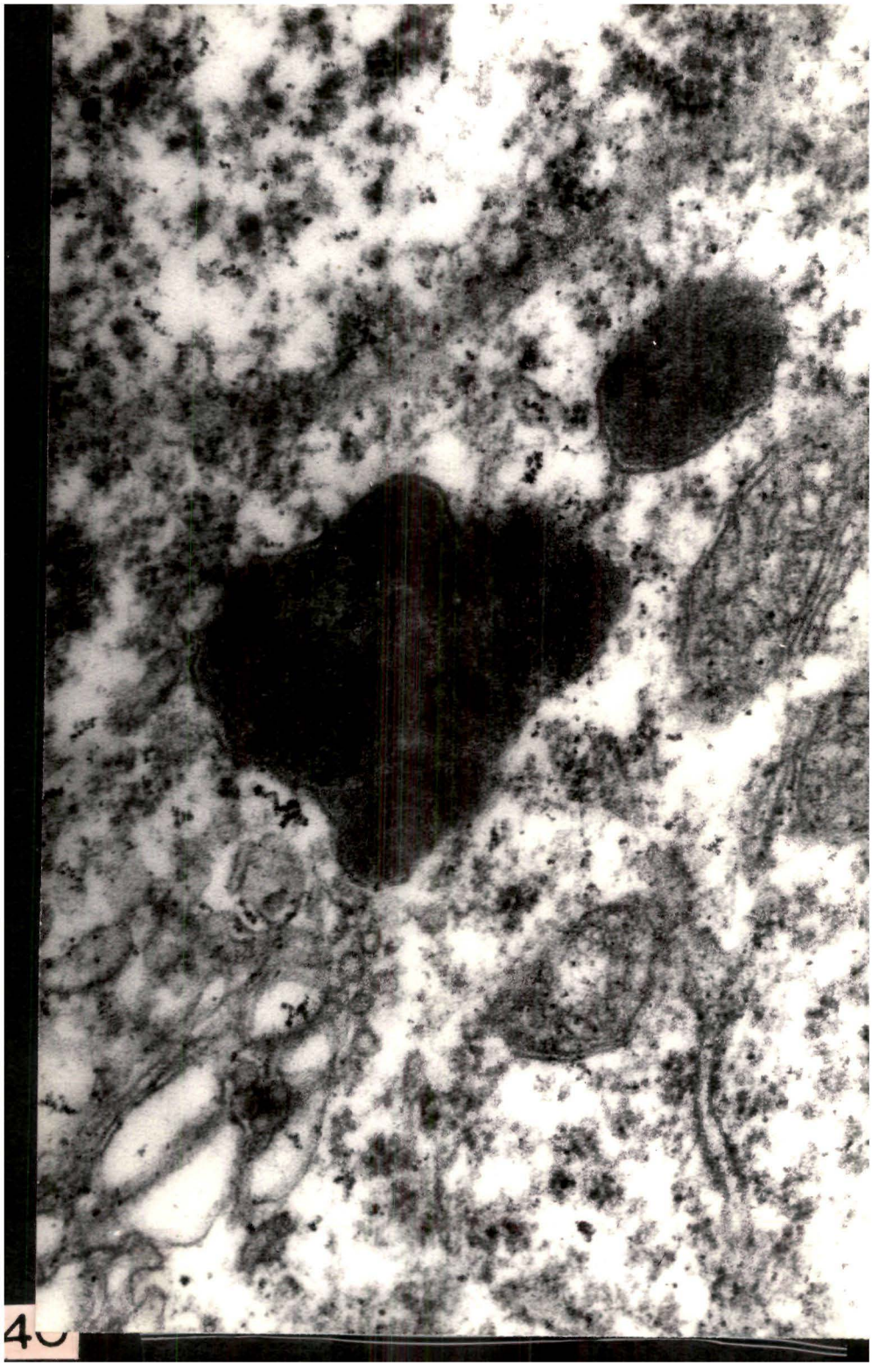
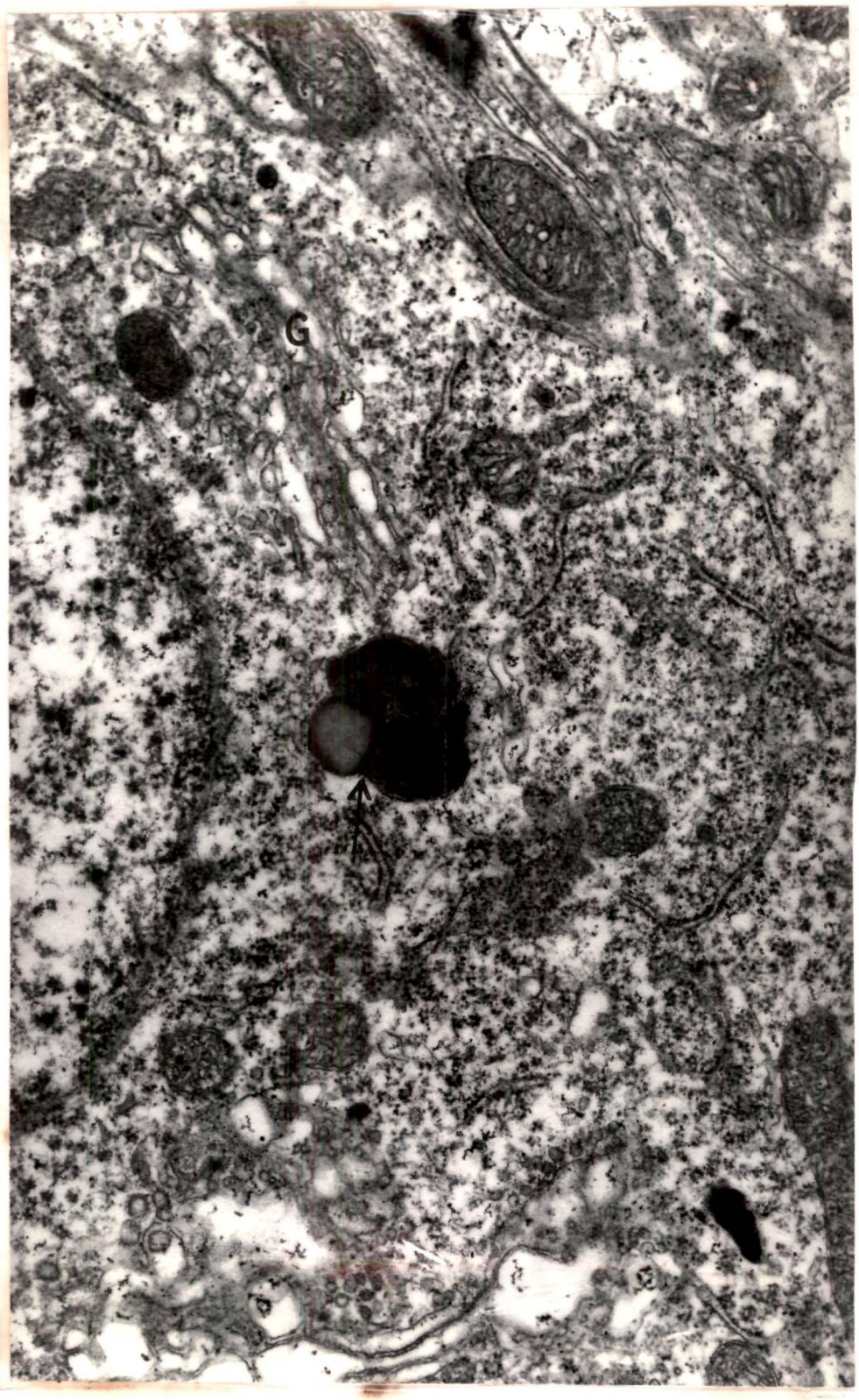
Explanation of Figure:

Fig. 40 : Electron micrograph, still higher magnification of Fig. 39 showing two electron dense bodies in the cytoplasm (X 48,000).



Explanation of Figure:

Fig. 41 : Electron micrograph of a part of spinal cord neurone of organophosphate-dichlorvos treated rat. Note the electron dense body with electron lucid vacuole, Golgi apparatus showing dilated cisternae (X 36,000).



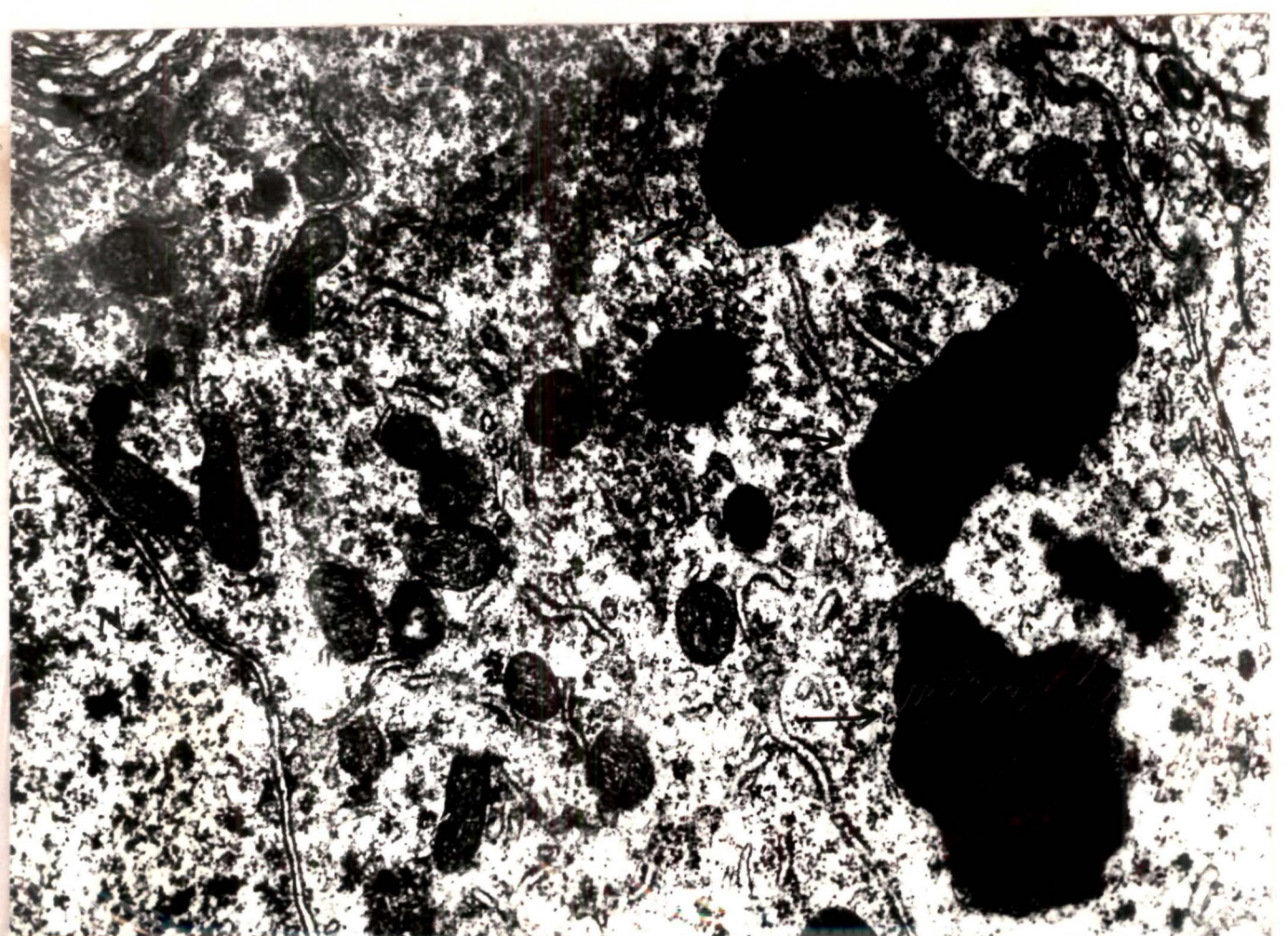
41

Explanation of Figures:

Fig. 42 : Electron micrograph of a part of cerebellar neurone of dichlorvos treated rat. Note three pleiomorphic electron dense bodies (arrows) and electron lucid vacuoles are also seen within the dense bodies (X 24,000).

Fig. 43 : Electron micrograph obtained from dichlorvos treated rat. Three polymorphic lipofuscin granules are visualized in the perikaryon of a cerebellar neuron. Note electron lucid vacuoles within the dense bodies (X 36,000).

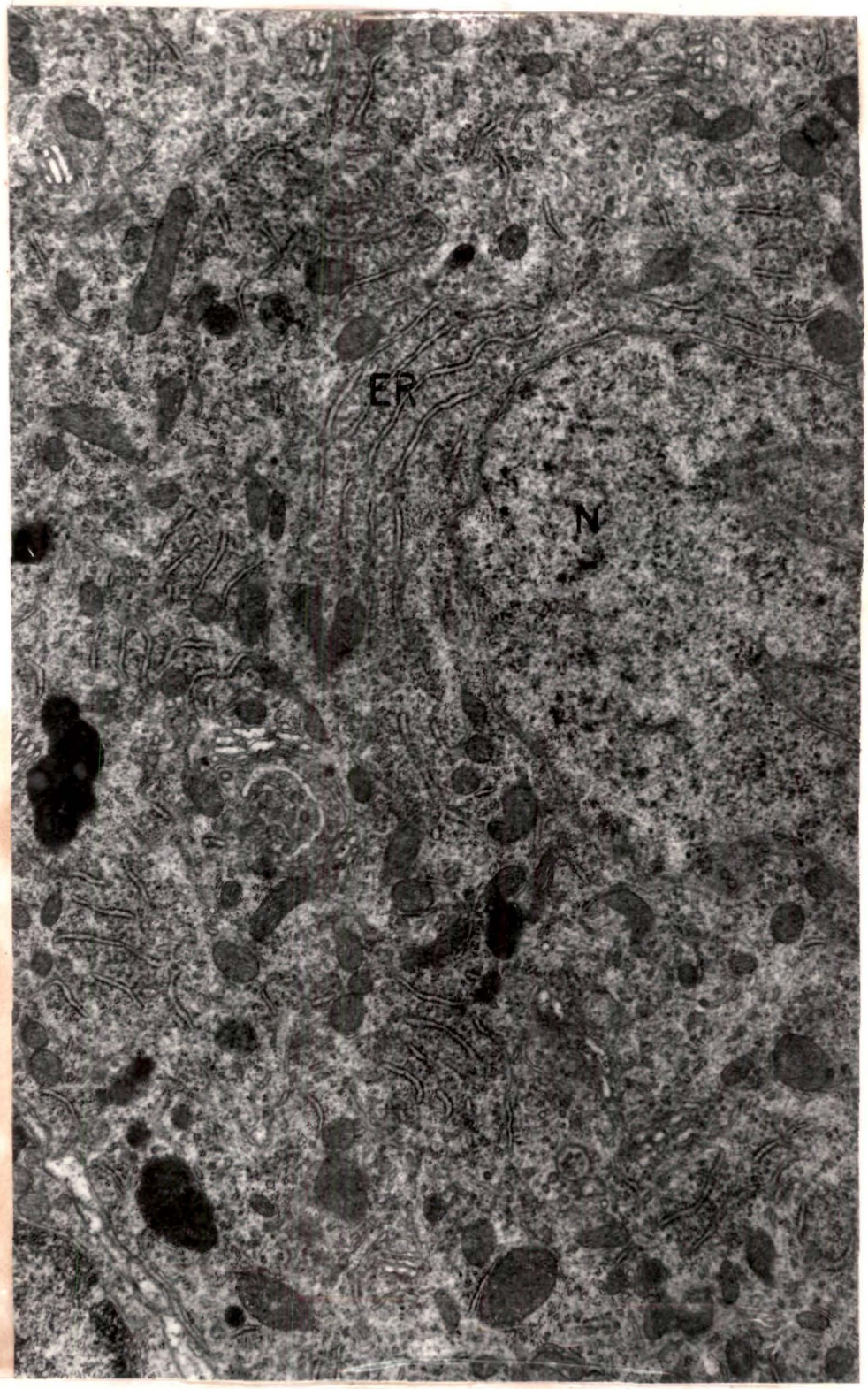
42



43

Explanation of Figure:

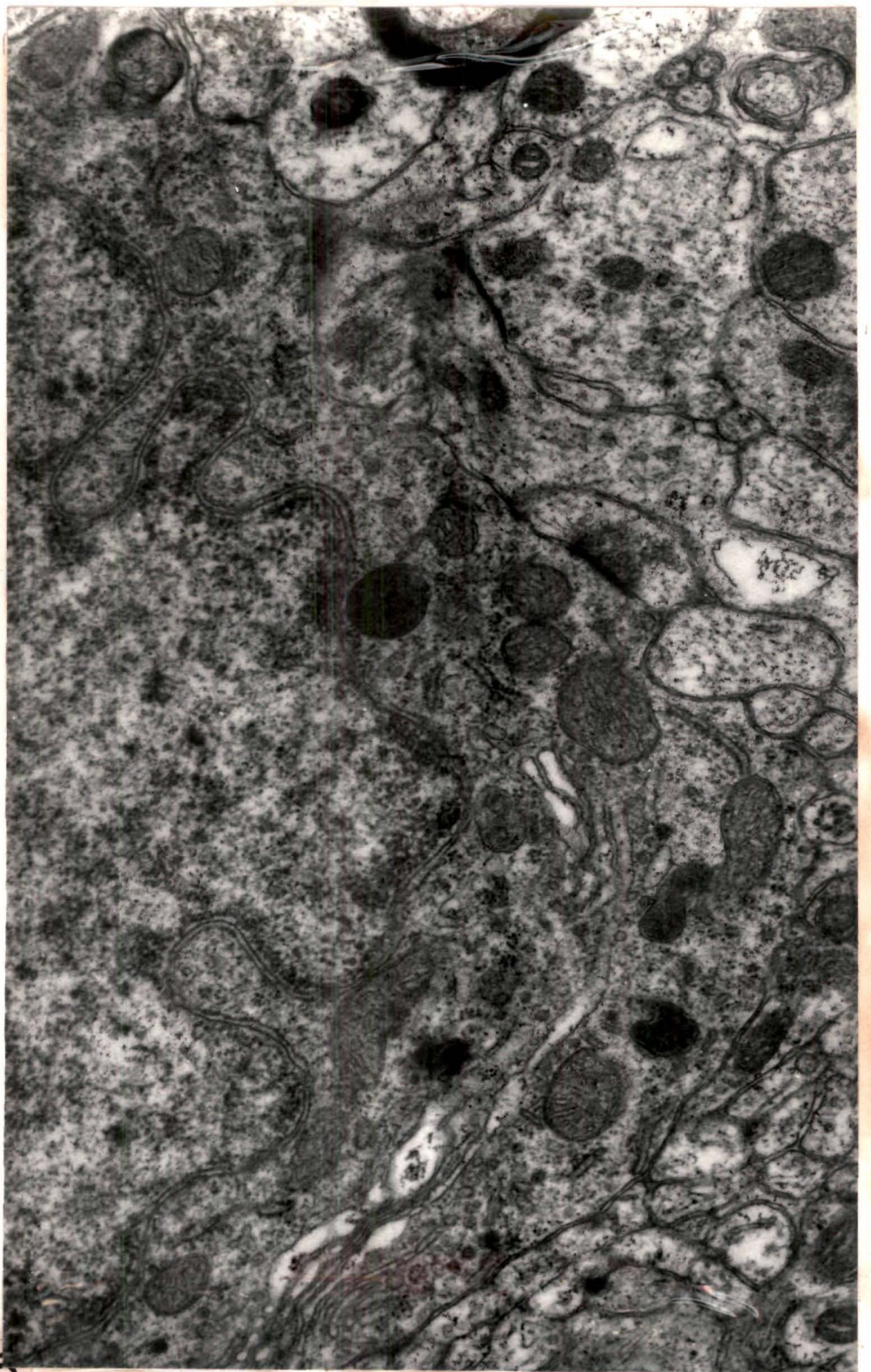
Fig. 44 : Electron micrograph of a part of cerebellar neurone of dichlorvos treated rat showing the large field of Fig. 42. Note the well preserved rough endoplasmic reticulum and mitochondria (X 16,200).



44

Explanation of Figure:

Fig. 45 : Electron micrograph, a part of a cerebellar neurone of organophosphate-dichlorvos intoxicated rat. Note the wavy contour and invagination in nuclear profiles and dilated cisternal of smooth endoplasmic reticulum (X 36,000).



45

Explanation of Figure:

Fig. 46 : Electron micrograph, an oligodendrocyte of dichlorvos-intoxicated rat cerebellum showing an indentation of the nucleus (N) and golgi zone (G) in its immediate vicinity (X 22,200).


N
G
46

Explanation of Figure:

Fig. 47 : Electron micrograph of a part of hypothalamus, dichlorvos treated rat, showing axonal profiler full of spherical and cylindrical synaptic vesicles (X 36,000).

47

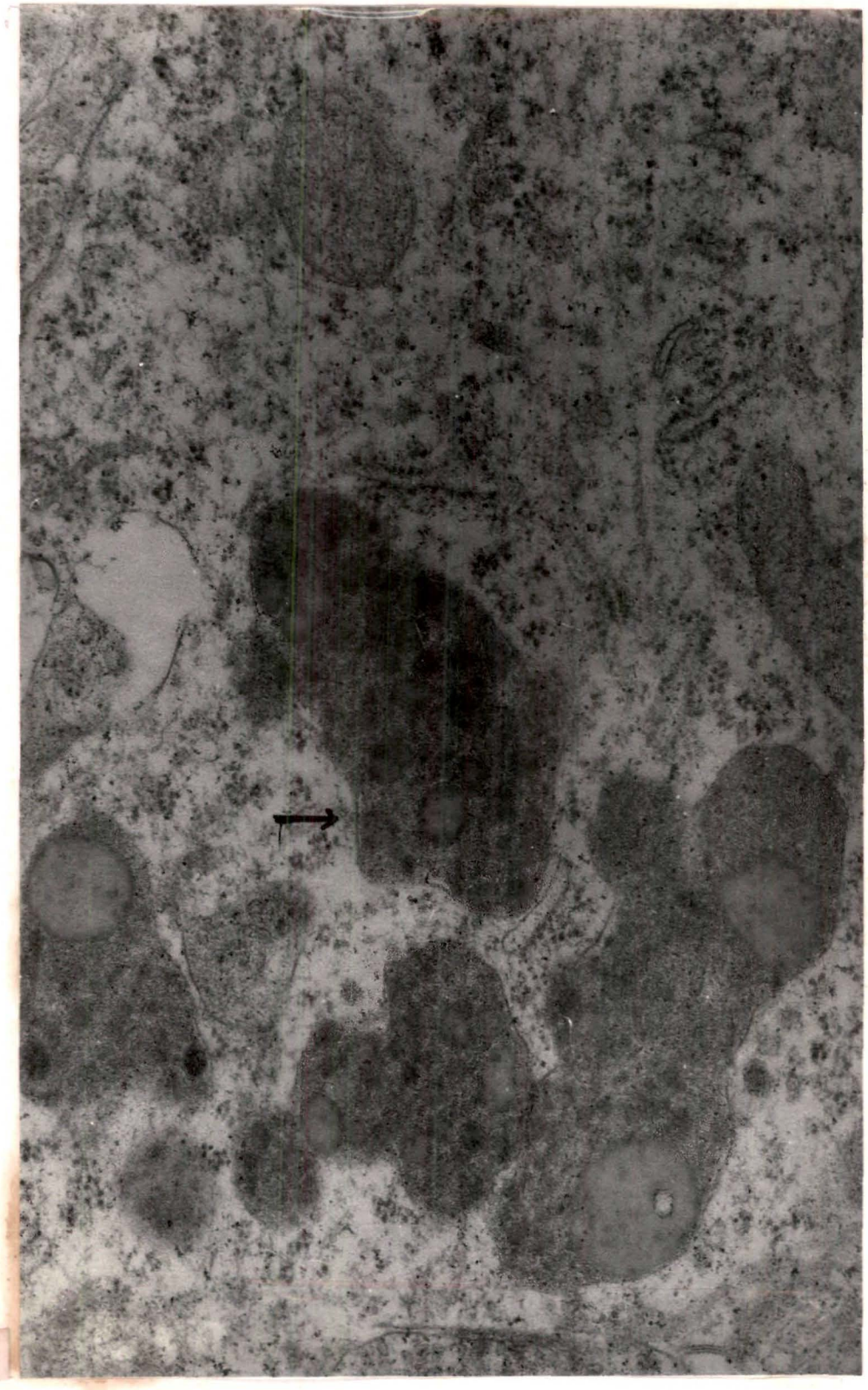
Explanation of Figure:

Fig. 48 : Electron micrograph of part of a hypothalamus from dichlorvos treated rat showing exhibiting evidence of oedema around an intact axonal profile (arrow) containing 3 well preserved mitochondria and numerous synaptic vesicles (X 54,000).

48

Explanation of Figure:

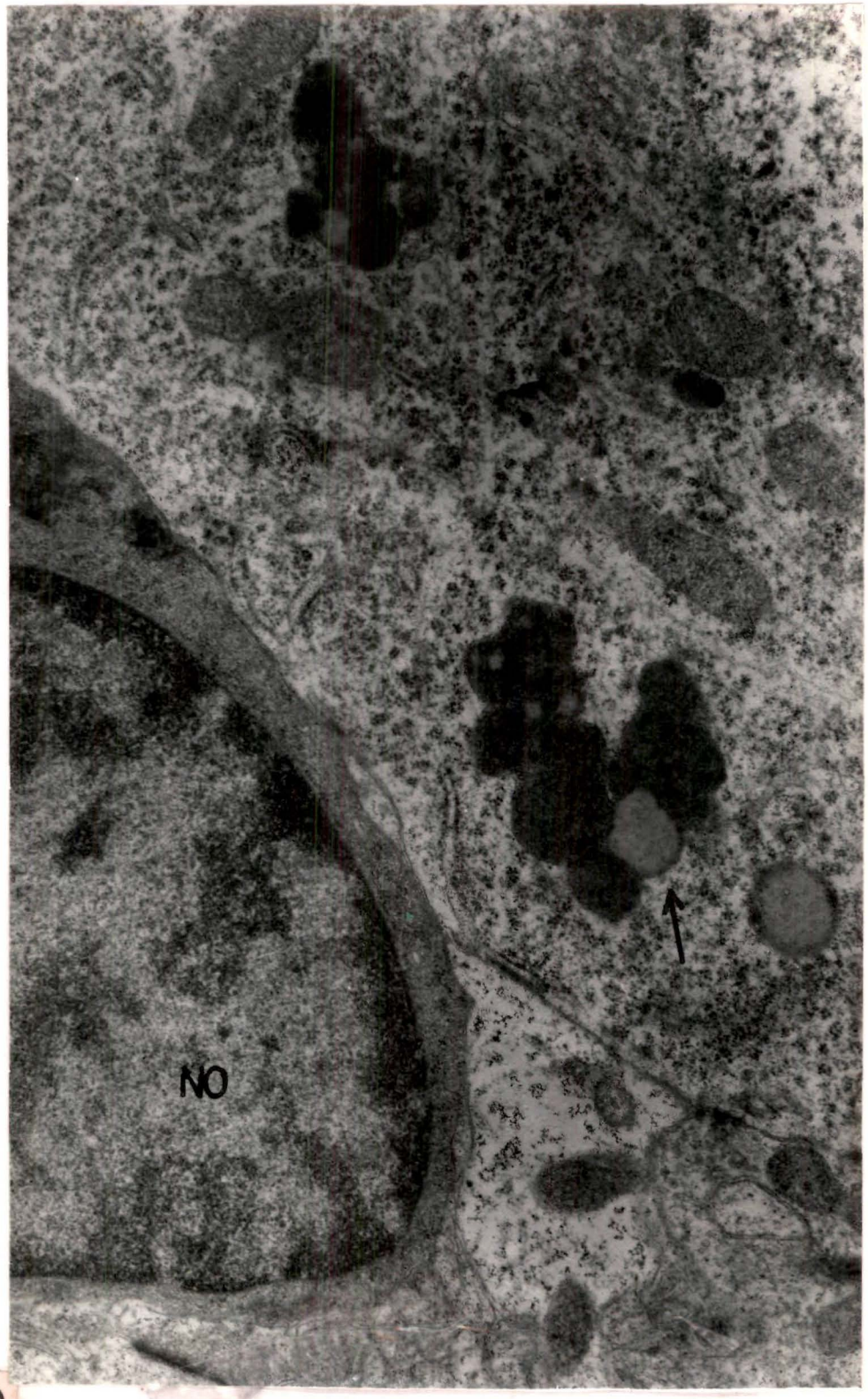
Fig. 49 : Electron micrograph, part of a nucleus of a hippocampal neuron and a dense body (arrow) limited by a single membrane are seen. Note the electron-dense granular profiles and an electron-lucid vacuole within the dense body (X 48,000).



49

Explanation of Figure:

Fig. 50 : Electron micrograph, part of a hippocampal neuron obtained from dichlorvos-intoxicated rat. A nuclear profile of an oligodendrocyte; Noteworthy is the vacuolated dense body (arrow) in its immediate vicinity (X 22,200).



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DISCUSSION

4. DISCUSSION

The results of the present study describe the preparation of two organophosphorus compounds and the neurotoxicological quantitative biochemical, neurohistochemical behavioural and electron microscopic investigation of various regions of the rat brain following organophosphate-'dichlorvos'-intoxication. The toxic effects of organophosphate pesticides are commonly ascribed to their ability to inhibit cholinesterases and to allow accumulation of acetylcholine at nerve-endings (Burns and Mand, 1965). Organophosphates are of particular interest because of their (i) increased application as insecticides to replace more environmentally and biologically persistent organochlorine pesticides and (ii) effects on nervous system of mammals (Koller et al., 1976). It is unfortunate that neurotoxicological alteration induced by dichlorvos has, so far, not been adequately investigated. Therefore, it would be appropriate to find out the effects of organophosphate-dichlorvos on different regions of the central nervous system.

4.1. Behavioural changes - correlation with biochemical findings:

The findings of the present study pertaining to the open field behaviour following daily administration of dichlorvos (3 mg/kg) indicate variable depressant effect on ambulation and rearing with a peak effect on the seventh day. The ambulation

was reduced to 88% of the mean on the seventh day and increased to 70% on the tenth day. This corresponds well with the peak decrease in amine concentration in different parts of brain on the seventh day and subsequent recovery on the tenth day. Rearing showed maximum depression of 98% on the seventh day which, however, increased to 82% of the mean on the 10th day. This correlates well with the concept that brain dopamine plays on important role in the complex stereotyped behaviour i.e. rearing (Kulkarni and Pandiya, 1972) and also with the dopamine content, particularly of the brain stem estimated on the 3rd, 5th, 7th and 10th day after dichlorvos in our studies (Tables 12-14). Preening is a behavioural response which is said to decrease with drugs causing stimulation of ambulation and rearing and vice-versa. In our findings preening also showed depression to 72% on the 7th day but contrary to ambulation and rearing, it showed a further decrease to 86% on the tenth day. This suggests that preening response has no correlation with the dopamine, norepinephrine and 5-HT levels in the brain. Depression of preening response has been ascribed to be a behavioural correlate of cortical stimulation and has been shown to be depressed by drugs like amphetamine and antidepressants (Gupta et al., 1971b). In our experiments a CNS depressant organophosphate-dichlorvos is simultaneously depressing all the open field parameters along with a decrease in the concentration of dopamine, norepinephrine and 5-HT. The defecation response sharply decreased on the seventh day. It may be presumed, in accordance with the hypothesis of Gupta et al. (1971a), that dichlorvos might be suppressing the nervous loci

of the posterior hypothalamus which control the reactivity of the sympathetic nervous system.

It is generally believed that norepinephrine level in the brain is responsible for the motor hyperactivity in rats under various experimental conditions (Matussek and Ruther, 1965). An increase in motor activity also occured after DOPA injection under certain conditions when dopamine levels rise in the brain, while norepinephrine increase is hardly significant (Goodwin et al., 1970). 5-HT and other catecholamine metabolites have also been accounted for increased motor activity (Scheckel et al., 1969). Our results on motor activity are well in accordance with these authors as the decrease in motor activity and depletion of levels of norepinephrine, dopamine and 5-HT run parallel. The gross and fine movements are equally and simultaneously affected as compared with the placebo. The motor activity starts returning to normal after a peak effect on the seventh day. This corresponds with the studies on fowls where it has been reported that dichlorvos has a transient and not a cummulative effect on CNS when given repeatedly over 2-3 weeks (Ciba-Geigy monograph, 1971). We have, so far, not come across any study in which brain levels of catecholamine and 5-HT have been estimated after dichlorvos in rats. In the present study dichlorvos significantly reduced dopamine, norepinephrine and 5-HT in different regions of the brain. This reduction may be due to a central cholinergic excitation as a result of accumulation of acetylcholine due to cholinesterase inhibition caused by the dichlorvos (Tracy et al., 1960). Partly or wholly, inhibition of the rate limiting enzyme,

tyrosine hydroxylase, may also be responsible for the suppression of NE and DA concentration as produced by physostigmine (Carlsson, 1974). Suppression of the monoamine levels in the rat brain seem to be in correlation with the behavioural depression and simultaneous decrease in amine concentration on the seventh day and subsequent recovery of both led this investigator to think positively on this pattern. Other studies on dichlorvos in experimental animals showed that cholinesterase inhibition is maximum on the seventh day and also that the effect of dichlorvos on nervous system in rats was transient and not cumulative when the drug was administered daily for two to three weeks (Ciba-Geigy Monograph, 1971). These findings are in correlation with the present results and may explain the recovery phase after seventh day of dichlorvos treatment.

4.2. Activity of Acetylcholinesterase:

It is well known that the organophosphorus poisons do not inhibit acetylcholinesterase uniformly in all parts of the brain (Heath, 1961). However, in most investigation only the average inhibition of whole brain is discussed (Goldstein et al., 1958; Durham et al., 1959; Tracy et al., 1969; Witter et al., 1961; Polson and Tattersall, 1969). Acetylcholinesterase activity is distributed in a very characteristic, uneven pattern that differs strikingly from the regional variation and as with many other enzymes, the activities of this enzyme in brain tissue varies inversely with brain size or body size respectively (Friede, 1966). Nachmansohn (1939) noted that acetylcholinesterase activity in the

brain of various species decrease as brain mass increases. Recently Teichert et al. (1976) have described the effect of acute and chronic treatment with dichlorvos on rat brain cholinergic parameters. They reported that the activity of acetylcholinesterase of whole brain decreased by about 70% after 1 hr oral administration of 50% of the LD_{50} and by 45% after the 14 days of daily administration of 5% of the LD_{50} (Teichert et al., 1976). The present investigation show that the depletion of acetylcholinesterase, is carried out in three different areas of the brain and spinal cord and reveals the regional characteristics of dichlorvos-intoxication. This inhibition of AChE is used for the index of neurotoxicity for further biochemical investigation after injecting the organophosphate dichlorvos. In mammals cholinergic stimulation by organophosphates results from the inhibition of acetylcholinesterase, the only biochemical or biophysical lesion implicated in almost all kinds of poisoning (Carida, 1964). Intoxication of pregnant rats with dichlorvos markedly inhibits their blood plasma cholinesterase without essentially affecting the blood cholinesterase and brain AChE in the offspring at various stages of ontogenesis (Zalewska et al., 1977). In contrast to other organophosphorus compounds, dichlorvos produced an increase in acetylcholine which was evident only in the hemisphere structures and not in the whole brain (Teichert et al., 1976). It remains to be determined if the action of dichlorvos on striatal acetylcholine is due to the more rapid turnover of acetylcholine in this area (Trabucchi et al., 1974) leading to greater accumulation, or whether the striatum is the key site for the neurotoxic action of the

dichlorvos. In the pure state, parathion, a phorphorothionate ester, is a poor inhibitor of the cholinesterase enzymes (Fukuto, 1957; O'Brien, 1960; Heath, 1961; Menzie, 1966) because of the deactivating effect of the thionate sulfur atom. By contrast paraxon, the corresponding phorphate ester of parathion, is a very potent anticholinesterase, similarly in case of dichlorvos, trichlorfon itself is devoid of anticholinesterase activity, whereas dichlorvos is a very strong inhibitor. In true sense, this reaction should not be considered a metabolic reaction, since the rearrangement occurs spontaneously and rapidly at neutral or alkaline PH (Fukuto and Metcalf, 1969).

4.3. Amino acid estimation:

In view of the reported diminution of amino acid concentration of brain areas in cases of convulsive disorders, including drug-induced convulsions (Tapia et al., 1967; Kar and Kateen, 1974), it was considered appropriate to investigate the effect of organophosphates on the free amino acid concentration of different regions of the rat brain. Investigations were carried out in three different areas of the brain to reveal the regional characteristics of dichlorvos-intoxication. Earlier, Singh and Malhotra (1964) had shown regional variation in the amino acid content of the monkey brain. Following intravenous injection of reserpine, they reported different changes in different amino acid levels of several areas of the brain. More recently Cutler and Dudzinski (1974) have described regional variations in amino acid content in the developing rat brain. Our findings, that the

amino acid concentration shows a regional heterogeneity in its distribution in the brain is of practical significance. The regional distribution of amino acids may have implications with respect to their functional role as neurotransmitters. It is well known that the anticholinesterase organophosphates, such as dichlorvos, can provoke marked alterations in the central nervous system (Modak et al., 1975). The results further show significant lowering of the concentration of taurine, GABA, glycine, phenylalanine, aspartic acid and lysine in different regions of the rat brain following dichlorvos administration. Significant changes in the underlying processes of their synthesis, uptake or degradation in different regions of the brain. Free amino acids are of considerable interest as the source from which proteins and neurohumours are synthesized and to which end-products degradation return. They also participate in the regulation of metabolic homeostasis, are part of ionic environment and act as a substrate for oxidative phosphorylation (Friede, 1966). It is likely that the marked alterations in the central nervous system - due to dichlorvos (Modak et al., 1975) are in some way related to these reductions in the level of amino acid in the different regions of the brain.

On the principle that the brain very often functions through "inhibition of inhibition" or "disinhibition", instead of direct stimulation, a search has been carried out by many workers for a deficiency in some inhibitory neurotransmitters in cases of convulsive disorders and epilepsy (Barbeau and Donaldson, 1974). Van Gender et al. (1972) have observed lowering of GABA and

aspartic acid levels throughout the cerebral cortex and of glutamic acid and taurine at the site of maximum seizure activity in patients suffering from epilepsy. In the present study the level of taurine, but not those of GABA and aspartic acid, showed a significant reduction in the cerebral hemisphere, suggesting that the reduction in GABA and aspartic acid level as reported by Van Gender et al. (1972) is small and highly discretre, perhaps localized to the cerebral cortex alone, and the reduction in taurine level is large and widespread. The latter suggestion is further supported by the observation that taurine levels have been significantly reduced in all the brain areas included in this study. Significant reduction of taurine in all the regions of the central nervous system appears to be the most characteristic feature of dichlorvos-intoxication. Taurine is an aminosulfonic acid possessing strong inhibitory effect when applied microiontophoretically to central neurones (Ehinger, 1973). Furthermore, Schmid et al. (1975) are of the opinion that the high affinity uptake of taurine in synaptosomal fractions of rat cerebral cortex is unique and highly specific. No specific action of several centrally acting drugs on the taurine uptake into synaptosomal fractions has, however, been observed by Schmid et al. (1975). For various reasons Barbeau and Donaldson (1974) favour a role for taurine as a stablizer of membrane excitability rather than as a neurotransmitter. In any case, the significant reduction of taurine following dichlorvos-intoxication deserves consideration.

Like taurine, also GABA and glycine are inhibitory transmitters (Curtis, 1974). A decrease in their levels is likely to be associated with hyperexcitability of neuronal structures. The reduced levels of GABA and glycine in the brainstem and spinal cord and of taurine in the cerebrum, cerebellum, brain stem and spinal cord, obtained in the present study, contribute to the hyperexcitability of these structures during dichlorvos-intoxication. On the contrary, aspartic acid is an excitatory transmitter (Curtis, 1974). It is associated with cells that mediate polysynaptic excitation and can excite the neurones receiving inputs from these interneurones (Apriron and Werman, 1968). A decrease in its level in the cerebellum, brain stem and spinal cord, recorded in this study, should, in the same way, result in neuronal depression. It need further investigation to determine whether this neuronal depression is primarily a part of "inhibition of inhibition". Interestingly, Tapia and Sandoval (1974) have ascribed another possible role to GABA. Their results seem to be consistent with the hypothesis that GABA is involved in the regulation of protein synthesis in brain 'in vivo'. It is suggested by the authors that GABA levels at the synaptic junctions might be more important for the regulatory role than its concentration in the whole brain.

Apriron and Werman (1968) have classified serine along with "inactive" amino acids and lysine as "untested". The latter is an essential dietary amino acids which is metabolically inert in animals as compared to the other amino acid (Meister, 1965). Since there are regional and individual variations in the response of neurones to amino acids, a renewed search for cells responding

to the apparently "inactive" or "untested" amino acid, serine and lysine, would be desirable. All the amino acids, active or inactive, according to Whittaker (1968), have similar subcellular distribution. On the other hand, interconversion of amino acid is also known to occur. According to Meister (1965), not only interconversion of serine and glycine occurs but also they participate in a number of reactions, including formation of tryptophan, cystathionine and cysteine. In our study serine was the only amino acid whose level did not show significant reduction in any of the regions of the brain and spinal cord. It is likely that the serine dependent functions are not disturbed by dichlorvos intoxication. On the other hand the fact, that the cholinesterase which is inhibited by organophosphates has a "serine" residue in its active centre that plays an essential role in the catabolism, should not be overlooked. The electron deficient phosphorus atom of the organic phosphorus compounds is known to attack the electron rich oxygen atom of the "serine residue" (Aldridge, 1964). Furthermore, the covalent phosphorus-serine bond is maintained long after the parent compound has disappeared (Coppage et al., 1975). This may play a role in keeping the serine level unchanged following dichlorvos intoxication. Of particular interest is, however, the significant reduction of phenylalanine level in the brain stem and spinal cord, labelled phenylalanine has been shown by Meister (1965) to be a precursor of epinephrine and norepinephrine. Further investigation of dichlorvos intoxication on the catecholamine level of discrete areas of brain showed the regional variation in our studies. In conclusion, it can be stated that the diminution of several

amino acids in discrete areas of brain following prolonged administration of dichlorvos might be contributing to its toxic effects. The characteristic reduction in the concentration of amino acid, particularly of taurine, can very well be utilized for the evaluation of the toxic effects of dichlorvos.

Levels of Monoamines:

The present investigations indicate a regional heterogeneity in the level of dopamine, norepinephrine and 5-HT after the experimental dichlorvos toxicosis. Whereas the concentration of dopamine was significantly lowered in the cerebral hemisphere, cerebellum and brain stem, the norepinephrine level showed a reduction in the cerebral hemisphere alone. On the other hand, the concentration of 5-HT was significantly increased in the spinal cord. In the cerebral hemisphere and brain stem, however, the level of 5-HT showed a significant reduction. Interestingly, Freedman and Giarman (1962) and Schanberg and Giarman (1962) have emphasized the point that these levels reflected the net results of numerous component processes. A considerable body of anatomic, pharmacologic and neurophysiologic evidences link central serotonergic mechanisms to the regulation of such diverse functions as sleep and body temperature, as well as to the pathophysiology of human ills ranging from Schizophrenia to Parkinsonism (Chase, 1974). The present observations, showing depletion of NE in the cerebral hemisphere and increased levels of 5-HT in the spinal cord, find a parallel in an earlier report of Freedman et al. (1970) who showed that 90 min after injection of psilocybin, levels of norepinephrine were decreased by 24% ($P < 0.001$) while 5-HT

levels were increased.

Changes in the level of brain dopamine are known to be associated with certain motor dysfunctions (Hornykiewicz, 1966). Also Freed et al. (1976) have shown a marked lowering of dopamine level of striatum after Mipafox (N,N-Di-isopropyl phosphorodiamidic fluoride) toxicosis. The reduced level of dopamine may be due to an increase in the turnover of dopamine itself which is induced by prolonged cholinergic stimulation (Corrodi et al., 1967). Furthermore, Freed et al. (1976) have postulated that the neurochemical imbalance produced as a result of inhibition of cholinesterase and reduction of dopamine may be partly involved in the delayed neurotoxic effects of certain organophosphorus compounds. Also, Fiscus and Van Meeter (1977) have found that maintained cholinesterase inhibition, following parathion intoxication, decreased endogenous levels of NE and DA in the cerebral cortex and increased turnover of NE in cerebral cortex and brain stem. In addition, DA turnover was found to be increased in corpus striatum but decreased in cerebral cortex. From our investigation it may, therefore, be suggested that there is a significant disturbance in the metabolism of catecholamines and 5-HT in dichlorvos toxicity.

4.5. Rate of Lipid-peroxidation:

As earlier our results indicate dose dependent inhibition of acetylcholinesterase activity following organophosphate-dichlorvos administration. This index of neurotoxicity finds

support in the observation of dose dependent increase in the rate of lipid peroxidation. The amount of malonicdialdehyde produce, as measured by thiobarbituric acid (TBA) assay, has been shown to be a true indicator of endogenous lipid peroxidation (Tappel and Walkin, 1960). The brain homogenate has apparently the necessary unsaturated fatty acids and the catalysts for peroxidation in the architecture of the cell itself which are readily available for reaction with molecular oxygen to undergo lipid peroxidation. Peroxidation involves the direct reaction of oxygen and lipid to form free radical intermediates and to produce semistable peroxides. Lipid peroxidation is damaging because of the subsequent reactions of free radicals, mainly peroxy radicals, that are produced (Tappel, 1970). Biomembranes and subcellular organells are the major site of lipid peroxidation damage. Mitochondria and microsomal membranes contain relatively large amounts of polyunsaturated fatty aids in their phospholipids. These include fatty acids with 2,4,5 and 6 double bonds for which relative rates of peroxidation both 'in vitro' and 'in vivo' are 1,4,6 and 8 respectively (Witting, 1965). Atleast two systems are important in the animal body to protect against membrane damage resulting from uncontrolled lipid peroxidation. These systems rely on selenium and vitamin E respectively and form the basis for hypothesis concerning the antioxidant functions of these nutrients (Combs et al., 1975) Hoekstra (1975) has shown that the organophosphate, tri-o-cresyl phosphate, interferes with selenium and glutathione peroxidase. It appears likely that a similar mechanism operates in the dichlorvos-induced increase in the rate of lipid peroxidation observed in the present investigation. Lipid peroxidation damage to mitochondria can have profound

effects on the cell and correlated well with the swelling and finally lysis and disintegration of mitochondria (Tappel, 1970). He also showed that this deterioration of isolated mitochondria can be conveniently measured by oxygen absorption or by the thiobarbituric acid reaction, and that the reaction can be inhibited by addition of vitamin E or ubiquinol both of which have lipid antioxidant activity. The similarity of peroxidation and products to electron dense pigment granules has been verified by Ohio et al. (1969). On the basis that lipid peroxidation of subcellular organelles gives fluorescent products with fluorescence and excitation spectra similar to those of lipofuscin pigments. Formation of the pigments appears to involve peroxidation of polyunsaturated lipids of subcellular membranes. Malonaldehyde, a major product of peroxidation of polyunsaturated lipids, reacts with primary amino groups of amino acids and proteins in a cross-linking reaction (Ohio et al., 1969). Of importance to cellular damage is the lability of lysosomal membranes to rupture with concurrent release of an array of hydrolytic enzymes with capacity to initiate cellular digestion and catabolism (Tappel, 1970). The increased incidence of pleomorphic electron dense bodies in our electron microscopic findings appears to be the end result of the increase of lipid peroxidation consequent upon dichlorvos-intoxication.

4.6. Histochemical investigation:

Acetylcholinesterase activity was inhibited in the cerebral cortex. This was also confirmed by the quantitative biochemical estimation following three different doses of organophosphate

dichlorvos. This inhibition of AChE is used as an index of neurotoxicity for further histochemical as well as biochemical changes in different regions of the brain. Activities of cytochrome oxidase and succinic dehydrogenase showed perceptible inhibition in the dichlorvos treated material. Both these enzymes belong to oxidative group and are located in mitochondria. According to Packer et al. (1967), only mitochondria possess mechanism for direct reaction with oxygen. In our finding these enzymes were inhibited which is also supported by the biochemical results showing an increase in the rate of lipid peroxidation. Tappel (1970) correlated well the rate of lipid peroxidation with swelling and finally lysis and disintegration of mitochondria. Recently, Malewska et al. (1977) showed the activity of succinic dehydrogenase and cytochrome oxidase of the brains of the rat foetus during ontogenesis, following intoxication of the mother with dichlorvos. Application of 2 and 10 LD_{50} doses of dichlorvos led to a decrease of the co-activity by about 20% and 40% respectively (Malewska et al., 1977). The present ultrastructural observation pertaining to morphological changes in mitochondria are consistent with the already known inhibition of mitochondrial enzymes. Furthermore, the increased incidence of lipofuscin pigments correlates well with the elevation of the rate of lipid peroxidation following dichlorvos intoxication.

4.7. Electron microscopic alteration:

The occurrence of variegated cytoplasmic electron-dense bodies was the main evidence of dichlorvos-intoxication. Some

of them are single membrane-limited bodies exhibiting uniform electron density and appear to resemble lysosomes. Whereas many others show electron lucent vacuoles and appear composite in nature. But in a few instances, double membrane dense bodies are also observed. The latter might possibly represent altered mitochondria. Furthermore, it is possible that cell injury triggers off a series of events culminating in pigment deposition (Maran and Glees, 1972a). Increased formation of lipofuscin pigment after neurotraumatization has already been reported by Hutter et al. (1966) and Merenyi et al. (1968). Also Lentz (1959) observed unequivocal deposition of lipofuscin, together with the appearance of giant mitochondria, within 3 days of limbs amputation in salamanders. Recently, Spoerri and Glees (1979) have reported increased accumulations of lipofuscin granules of various sizes and shapes associated with altered mitochondria following TOCP poisoning of avian spinal ganglia. The changes in the neuronal cytoplasmic induced by organophosphates are considered to be proliferated, representing either a direct effect of the organophosphate or a secondry response to a primary toxic effect on the neurites (Wisniewski et al., 1968 and Le Vay et al., 1971). It has been suggested that neurotoxic effects on cells is not due to blocking of axonal flow, but rather to an increased rate of synthesis of filaments (Le Vay et al., 1971). Proliferation of ER in some cells reflected in the parallel increased in protein synthesis is followed by proliferation of filaments in other neurons. Several reports demonstrating typical mitochondrial degeneration following TOCP poisoning are available

(Ahmad and Cleer, 1968; Ahmad, 1970; 1971; 1973 and Ahmad and Cleer, 1976). Furthermore, Spoerri and Cleer (1979) also provided ultrastructural evidences for the involvement of mitochondria in the formation of osmophilic pigment or neuronal lipofuscin, a concept advance by Haran and Cleer (1972), Spoerri and Cleer (1973; 1974) and Cleer et al. (1974). This concept assumes that pigment formation is a metabolic product inherently related to mitochondrial deterioration, irrespective of the primary cause being either aging or intoxication.

Lipofuscin like pigments in man and animals, either experimentally produced in the latter or those primarily associated with specific local or environmental factors in both, are generally classified under the heading "ceroid" (Porta and Hartroft, 1969). This term is now commonly applied to lipo-pigments induced by a number of experimental conditions (Cleer and Haran, 1976). It is considered to be the intermediate stage of auto-oxidising lipid and lipofuscin. Recently, Haran et al. (1977a) have reported the thallium-induced increase of similar dense bodies in the rat area postrema and hippocampus (Haran et al., 1977b). According to Kerenyi et al. (1963) these pigment cells are irreversibly injured lysosomes and are likely to develop under the influence of a specific stress on the nervous system. Cleer and Haran (1976), on the basis of the available experimental evidences, concluded that neurones subjected to enhanced or reduced metabolic activity respond in final stages by lipofuscin/ceroid formation. Furthermore, they have shown that the main material for lipofuscin stems from

degenerating mitochondria. Enhanced cellular metabolism leads firstly to an increase in mitochondria and due to the limited life of mitochondria to their greater turnover. The residual lipids and proteins of mitochondria are transferred by lysosome to lipofuscin/ceroid, which can be further metabolised if the over all metabolic condition of the cell is sufficient. But neurones may be overloaded and be incapable of lipofuscin removal.

It is of interest to note that the origin of lysosomes from the Golgi apparatus has been claimed by Novikoff (1967). On the basis of his combined histochemical and electron microscopical observations in nerve cells. He has described a so-called "Gerl" complex which represents Golgi apparatus-endoplasmic reticulumlysosome. According to this hypothesis, the lipofuscin pigment may be formed as a result of a chain of activity in Golgi apparatus, endoplasmic reticulum and lysosomes. In the light of the above mentioned observations, the results of the present study can be interpreted as follows; Organophosphate-diehlorvos-intoxication, acting as a specific stress on the nervous system, showing degeneration in myeline, alteration in mitochondria thereby culminating in the excessive deposition of pigment in neuronal perikarya.

Following tri-cresyl phosphate intoxication, chromatolising spinal neurons were detected in the chick by Jamsik and Glees (1966). Furthermore, Ahmad and Glees (1971) reported the increased occurrence of laminated cytoplasmic inclusions bodies

in the spinal cord of tri-orthocresyl phosphate-intoxicated hen. They postulated that phospholipids become unmasked during a degenerative process and arrange themselves in the form of laminated dense bodies. It is conceivable that the increased incidence of the granular and vacuolated electron-dense bodies observed in this study might represent a disturbance in the phospholipids. In the present study, another interesting finding was the occurrence of dense particles in some of the hippocampal region as well as in the neighbouring oligodendroglicyte. Although vacuolated electron-dense bodies (or the residual bodies) are not ordinarily noticeable in the normal oligodendrocytes (Peters et al., 1970; Haran and Glees, 1972b), they were frequently encountered in the organophosphate dichlorvos intoxicated rats. It is more likely that single membrane-bound dense bodies represent dichlorvos induced alterations in the lysosomal apparatus. The functions of lysosomal apparatus include the uptake, digestion and defaecation of macromolecules and organelles (Dernick et al., 1976). It represents the major site of intracellular digestion. The primary lysosomes are known to fuse with vacuoles, such as phagosomes or autophagosomes, in which intracellular and extracellular macromolecules have been sequestrated for subsequent hydrolytic digestion (Novikoff, 1973). In these secondary lysosomes (or digestive vacuoles), the digestive enzymes and their macromolecular substrates are compartmentalised for catabolism. Sequestration of intracellular materials is accomplished by the process of autophagy. Despite the apparent capacity of lysosomes for extensive degradation of a wide variety of macromolecules, the lysosomes containing undigested residual material do occur. The extent to which exocytosis of these residual bodies occurs is not known (Novikoff, 1973). In a few instances, oligodendrocytes of the experimental material conformed

to the description of 'reactive oligodendrocytes' (Maxwell and Kruger, 1966). Pleomorphic electron-dense cytoplasmic bodies formed the chief characteristic of these oligodendrocytes. Dense-bodies of oligodendrocytes have also been identified as lipofuscin granules by Maxwell and Kruger (1966). But the rats used in this study were not older than 6 months, an age when lipofuscin is rarely discernible in the oligodendrocytes (Clees and Hasan, 1976). Interestingly, Mackey et al. (1964) have reported that neurones undergoing chromatolysis accumulate dense bodies. The two functions usually attributed to oligodendrocytes are the formation of myelin and the nutrition of neurones (Peters et al., 1970). It is tempting to correlate the accumulation of dense bodies in the oligodendrocytes with dichlorvos-induced neuronal chromatolysis, because in the latter case, nutrition of neurones is bound to suffer. The occurrence of variegated cytoplasmic dense bodies observed in the present study can thus be accounted for. However, Golgi apparatus, rough endoplasmic reticulum and ribosomes, do not exhibit significant response to organophosphate-dichlorvos-intoxication.

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1. S. Fatehyab Ali, Mahdi Haran and Arrifuzzaman (1977). "Observation on the organophorphate insecticide dichlorvos induced changes in the aminoacid and enzyme content of different regions of the central nervous system". Presented at Silver Jubilee Conference of Anatomical Society of India held at K.G's. Medical College, Lucknow from 29th Dec. 1976 to 1st Jan. 1977, J. Anat. Soc. India. 26(1): p. 14.

2. S. Fatehyab Ali and Mahdi Haran (1977). "Effect of organophorphate insecticide dichlorvos on the aminoacid content of different regions of the rat brain and spinal cord". Indian J. Expt. Biology, 15: 759-761.

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Index of abbreviations used in the description of electron micrographs

A - axone

C - capillary

CL - capillary lumen

D - dendritic process

Db - dense-body

E - endothelium

EM - electron micrograph

ER - endoplasmic reticulum

G - golgi-zone

L - lysosome

M - mitochondria

N - nucleus of neurone

NU - nucleolus

ON - oligodendrogliacyte nucleus

P - pericyte

Index of abbreviations used for labelling histograms

(Figs. 22, 23, 24 and 25)

ASP - Aspartic acid

GAB - Gamma aminobutyric acid

GLY - Glycine

LYS - Lysine

PHE - Phenylalanine

SER - Serine

TAU - Taurine